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Simonetta Giulia Papa

Candidate number: C4167355

**CHARACTERIZATION OF A
NANOPARTICLE DRUG DELIVERY
SYSTEM FOR THE TREATMENT OF
INFLAMMATION IN SPINAL CORD
INJURY**

IRCCS – Istituto di Ricerche Farmacologiche

Mario Negri, Milan

Thesis submitted for the degree of
Doctor of Philosophy

Life and Biomolecular Sciences
The Open University

September 2016

A. ABSTRACT

Spinal cord injury (SCI) results from a mechanical *primary injury* that is followed by a multifactorial *secondary injury* which worsens the clinical course. Persistent inflammation is a crucial event during the secondary injury. Indeed, microglia/macrophage respond to traumatic stimuli by adopting an activated phenotype, which has a dual role. An M1 phenotype, associated with harmful effects, is expressed very early and persists for a long time in the injured site, whereas M2, associated with a beneficial phenotype, has only a transient expression in a subacute phase of the trauma. This suggests that microglia/macrophage mediated inflammation is a crucial therapeutic target.

Nanoparticles (NPs) are selectively engulfed by microglia/macrophage as part of their endocytic/phagocytic activity in removing foreign bodies. Microglia assume phagocytic activity after traumatic stimuli and this makes NPs an excellent tool for carrying drug to these cells. So, in order to counteract this deleterious pro-inflammatory response, we decided to maximize the efficacy of a well-known anti-inflammatory drug, minocycline, through a NPs delivery tool selectively targeted to microglia/macrophage. We developed and tested a new drug delivery nanocarrier (minocycline loaded in poly- ϵ -caprolactone NPs, PCL Mino) *in vitro* and *in vivo*. Specifically, we demonstrated a reduced activation of microglia/macrophage after PCL Mino treatment *in vitro* and a reduction of cells with phagocytic-like phenotype, up to 15 days tested *in vivo*. To clarify the involvement of the inflammatory response

associated to microglia/macrophage in SCI progression, we treated SCI mice in acute and subacute phase. PCL Mino treatment, only when administered acutely after the damage, was able to ameliorate the locomotor activity up to 63 days post injury. Furthermore, we demonstrated that this treatment reduced M1 macrophages recruitment orchestrated by activated microglial cells via CCL2 chemokine in the damaged site, suggesting a deleterious effect in the early phase of the injury of these cells.

In conclusion, this delivery tool represents a new hope for SCI treatment by showing several advantages compared to conventionally administered anti-inflammatory therapy, such as maximization of therapeutic efficiency and reduction of side effects. Furthermore, the potential of transfer to clinical practice is aided by the large clinical use of minocycline and the high biocompatibility of the proposed NPs.

B. ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Pietro Veglianesi for the great academic and personal support given to me in all these years spent in his laboratory.

A sincere “thank you” goes to my supervisor, Professor John Priestley, for his important help and for his great availability during the period of my PhD.

A special thanks to all the former and current members of the Spinal Cord Injury and Neuroregeneration Unit, for making me grow professionally and, above all, for being friends.

I thank Dr. Gianluigi Forloni for giving me the chance to join his laboratory, the Mario Negri Institute for Pharmacological Research for giving me the opportunity to undertake my PhD and all the people who work here, for making me feel at home.

Finally, my most sincere thank goes to who believes in me and loves me. You make my life worth living.

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**D. LIST OF
ABBREVIATIONS**

BBB	Basso Beattie And Bresnahan
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BSCB	Blood-Spinal Cord Barrier
BMS	Basso Mouse Scale
CNS	Central Nervous System
CPZ	Chlorpromazine
CSF	Cerebrospinal Fluid
CSPG	Chondroitin Sulfate Proteoglycans
CST	Corticospinal Tract
DPI	Days Post Injury
EGFP	Enhanced Green Fluorescent Protein
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FGF-2	Fibroblast Growth Factor-2
GABA	Gamma-aminobutyric acid
GDNF	Glial cell-derived neurotrophic factor
IP	Intra-peritoneal
IV	Intra-venous
IT	Intrathecal
LPS	Lipopolysaccharide
LSFM	Light Sheet Fluorescence Microscopy
MAIs	Myelin associated-inhibitors

LIST OF ABBREVIATIONS

MP	Methylprednisolone
MRI	Magnetic Resonance Imaging
MVB	Multivesicular Bodies
NF	Neurofilament
NGF	Nerve Growth Factor
NP	Nanoparticles
PCL	Poly- ϵ -caprolactone
PLGA	Poly(lactic-co-glycolic acid)
PMMA	Poly-methyl-metacrilate
PNS	Periferal Nervous System
Qdot	Quantum dots
RAG	Regeneration Associated Genes
RhB	Rhodamine B
SC	Spinal Cord
SCI	Spinal Cord Injury
SD	Standard Deviation
SEM	Standard Error of Mean
TEM	Transmission Electron Microscopy

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F. INTRODUCTION

GENERAL INTRODUCTION

Spinal Cord Injury (SCI) represents the most frequent disabling injury between the diseases of the spine. An estimated 2.5 million people worldwide live with SCI and more than 180,000 new injuries are reported each year (Lee et al.; 2014). As a consequence of the injury, severe impairments or loss of motor and sensory function arise leading to tetraplegia or paraplegia (Becker et al.; 2003). Therefore, SCI has a great impact on the quality of life of the affected persons and also represents a heavy burden for the society in terms of loss of income and healthcare costs. Specifically, the devastating consequences following SCI are the outcome of a two-step process that initiates with a mechanical insult to the spinal cord (*primary injury*) and is followed by a wide spectrum of different events (extending from massive neuroinflammation reactions to the formation of a glial scar that inhibits axonal regeneration) that is called *secondary injury* (Borgens et al.; 2012). Because of the different neuropathological aspects that characterize secondary injury, SCI is classified as a multifactorial disease. To the present, high dose methylprednisolone (MP) administration is still the only recommended neuroprotective route to prevent further *secondary injury* damage (Sayer et al.; 2006). Unfortunately, the systemic high dose of MP also seriously aggravates adverse side effects such as wound infections, pneumonia and acute corticosteroid myopathy (Baptiste et al.; 2007) limiting the drug's

neuroprotective potential. Furthermore, it is demonstrated that MP shows only modest overall improvements in the neurological functions (Chvatal et al.; 2008). So, new therapies are still required to treat efficaciously the SCI pathology.

Much evidence shows that acute and chronic inflammation is implicated in the development and exacerbation of the *secondary injury* phase contributing negatively on outcome in SCI

Specifically, activation of microglia/macrophages and the associated inflammatory response appears to be a self-propelling mechanism that leads to progressive neurodegeneration. Therefore, microglia/macrophages are considered a crucial therapeutic target after SCI and a selective treatment of this cell population could represent an interesting strategy to counteract the spreading of the *secondary injury*.

Contextually, targeted drug delivery nanoparticles (NPs) could be a promising approach in term of selectivity, improved drug efficacy and reduced side effects. Indeed, NPs for biomedical applications has acquired a great importance in recent years representing a primary vehicle for drug delivery into phagocytic cells, such as activated microglia/macrophages.

In the present study, it will be characterized the use of a nanovector drug delivery system able to modulate the inflammatory reaction. In particular, a nanoparticle system composed of poly- ϵ -caprolactone and polyethylene glycol (PCL) will be loaded with a well-known anti-inflammatory drug, minocycline, to perform a targeted anti-

inflammatory therapy addressed to modulate microglia and macrophages activation. The proposed treatment will be also useful to clarify the involvement of the inflammatory response related to the activated microglia/macrophages during SCI progression

1. SPINAL CORD ANATOMY

The central nervous system is composed of the brain and the spinal cord (SC).

SC is the seat of recruitment, processing and transmission of information and provides a means of communication between the brain and the peripheral nerves.

The SC is made of a thick bundle of neurons and resides in the vertebral column, the latest composed of individual vertebrae, which acts as a protective barrier. SC has a cylindrical shape, with an average diameter of 8-10 mm, and extends from the *foramen magnum* at the base of the skull, seat of the first cervical vertebra (*atlas*), proceeding from the medulla oblongata up to the first lumbar vertebra (L1) (human) or the fourth (L4)(mice). Indeed, SC does not occupy the vertebral canal for its entire length, even though at the level of the sacrum there is the sacral cord, with a similar structure. At that level, the SC is cone-shaped (medullary cone) and continues caudally as a thin fibrovascular structure surrounded by the nerves of the cauda equina, anchoring the coccyx. SC is divided into several regions: cervical, thoracic, lumbar, sacral and coccygeal. Sensory information, carried by afferent axons of peripheral nerves, penetrates into the spinal cord via the dorsal roots. The motor functions, conveyed by the efferent axons, leave the SC through the ventral roots. Once the roots are joined, the sensory and

motor axons travel along and combine to form the **spinal nerves**, which exit the spine through the intervertebral holes.

The spinal nerves innervate most of the body and emerge from the spinal cord in the form of 31 pairs (34 pairs in mice), one on each side of the SC. In Table 1 is schematically shown the subdivision of the nerves depending on the regions of the SC.

SC region	Number of Nerves (human/mouse)	Innervation
cervical	8	Shoulder, hand, neck, arms
thoracic	12/13	Thorax
lumbar	5/6	Legs, hip
sacral	5/4	Legs, glutex, anus
coccygeal	1/3	Viscera, small basin

Table 1. Subdivision of peripheral nerves

The spinal cord is surrounded by the meningeal shrine, composed of three protective tissue membranes, which extends along the brainstem and the brain. Taken together, the three membranes are called **meninges**: the most outer layer of the meninges is called the dura mater, for its thickness and its consistency; the intermediate layer, arachnoid, is characterized by extensions branched off to reach the

underlying layer; the third layer, the pia mater, is a thin and delicate layer of cells which envelop the entire surface of the bone marrow. The space between the arachnoid membrane and the pia mater is full of cerebrospinal fluid that helps protect the marrow. As the pia mater adheres strictly to the cord, the arachnoid membrane has some points of dilation, called cisterns.

The main arteries that supply blood to the tissue run through the subarachnoid space, which for this reason is the bleeding site in case of trauma.

Observing a coronal section of spinal cord, it is possible to detect the provision of the gray matter, inside, and white matter, outside.

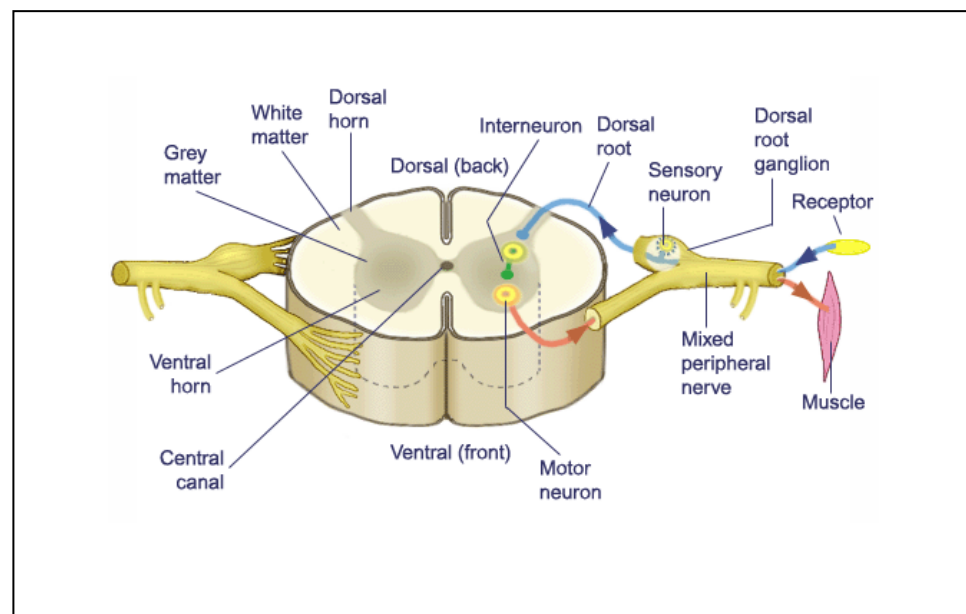


Figure 1. Internal organization of spinal cord

The **white matter** is organized in columns, called dorsal (or posterior), lateral and ventral (or anterior), each of which contains bundles of

myelinated axons with specific functions. The lateral columns are made up of axons which extend from the cerebral cortex until they contact the spinal motor neurons. The dorsal columns carry the ascending sensitivity information from the somatic mechanoreceptors. The ventral columns carry both ascendants information about the thermal sensitivity and descending motor information.

The **gray matter** of the spinal cord, divided into dorsal and ventral horns, is constituted by neurons carrying signals capable of putting in connection neurons localized in different levels of the spinal cord and the brain. The dorsal horn neurons receive sensory information through dorsal roots of the spinal nerves. The preganglionic neurons of the visceral nervous system are located in an intermediate region between the dorsal horn and the ventral horn.

The ventral horn contains the cell bodies of motor neurons that send axons to ventral roots reaching the striated muscles which are able to contract. The gray matter of the SC is divided into ten large groups of cells known as Rexed's laminae. Laminae I-VI are located in the dorsal horn, lamina VII is in the intermediate horn, and laminae VIII-X are in the anterior horn.

Laminae I-IV are delegated to the reception of stimuli from the skin, lamina V receives axons from the skin, muscles and viscera, and lamina VI receives proprioceptive fibers. The extensions of afferent fiber entering the spinal cord are divided into ascending and descending

axons that are located in the Lissauer tract that divides the apex of the dorsal horn from the back seam.

Spinal circuits

Motor activity is regulated by different groups of neurons, mutually linked by multiple synapses (Fig.2).

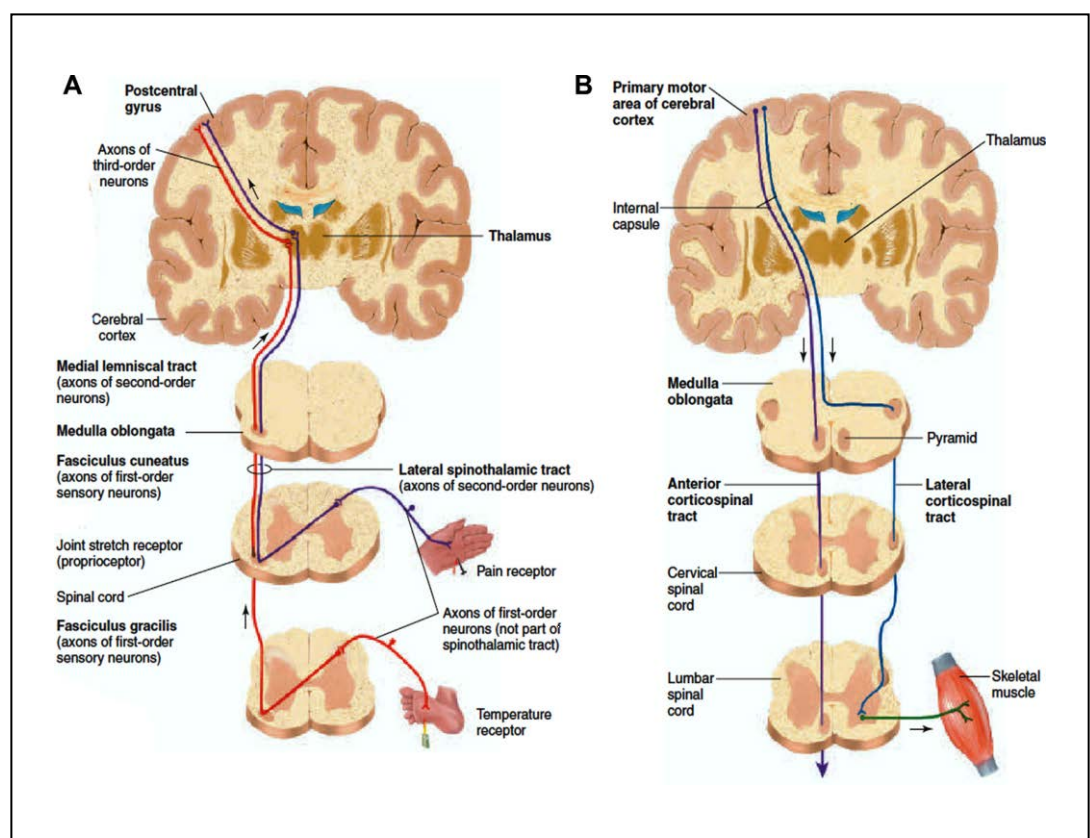


Figure 2. Organization of the nervous structures involved in mobility regulation (from Silva et al.; 2014)

The first group of nerve circuits is located in the gray matter of the SC, which cells include a **motor neurons** that innervate the skeletal muscle fibers and interneurons of the SC. All motor commands are transmitted to the muscles only through the a **motor neurons**. The interneurons

receive both sensory afferents and descendants projections from the upper centers and provide much of the reflex coordination among the different muscle groups.

The second group consists of descending pathways including the upper motor neurons, located in the brainstem and cerebral cortex, and able to connect interneurons and/or the **α motor neurons**. The descending pathways integrate the information necessary for the execution of elementary movements of the body government and postural adjustment.

The third group consists of the cerebellum, having the main function of coordinating the activity of motor centers able to carry out complex movements.

The other subset consists of the basal ganglia, important in the initiation of voluntary movements. The axons of motor neurons induce muscle contraction via neuromuscular junctions positioned between the muscle fibers and the motor neuron terminations. The release of the neurotransmitter acetylcholine stimulates the shortening of the muscle fiber.

2. SPINAL CORD INJURY (SCI)

Spinal cord injury is a disabling condition that affects sensory and motor functions and leads to devastating neurological deficits and impairment. SCI provokes the partial or total loss of sensory/motor capacity, leading to paraplegia or tetraplegia and other common problems such as frequent infections, cardiac and respiratory problems, vesical and bowel malfunction (Watson et al.; 2009).

All of these problems have an enormous impact on the life of SCI patients and represent also a heavy burden for society in term of health-care cost.

SCI affects about 180.000 new individuals each year for a total of 1,3 million persons worldwide (Lee et al.; 2014). In Italy there are about 70,000 people with spinal cord injury, of which 2/3 are younger than 60 years, with incidence between 1,500 and 1,200 cases per year. The therapies available today only bring modest benefits to the quality of patients life, and are not able to completely restore the motor functionality. It is therefore crucial to find new therapeutic strategies for the treatment of SCI.

2.1 Etiology

SCI can be caused by traumatic or non traumatic events.

Trauma can break, displace or collapse one or more vertebrae. The bleeding, inflammation and accumulation of cellular infiltrates create further damage, complicating the clinical picture. Non-traumatic events which may cause a spinal cord injury are: arthritis, cancer, inflammation, infection or degeneration of the intervertebral discs.

The most common causes of spinal cord injuries are accidents of various types (motor vehicle accidents, everyday falls, sports, work), as well as injuries from assaults (firearm wounds or scuffles).

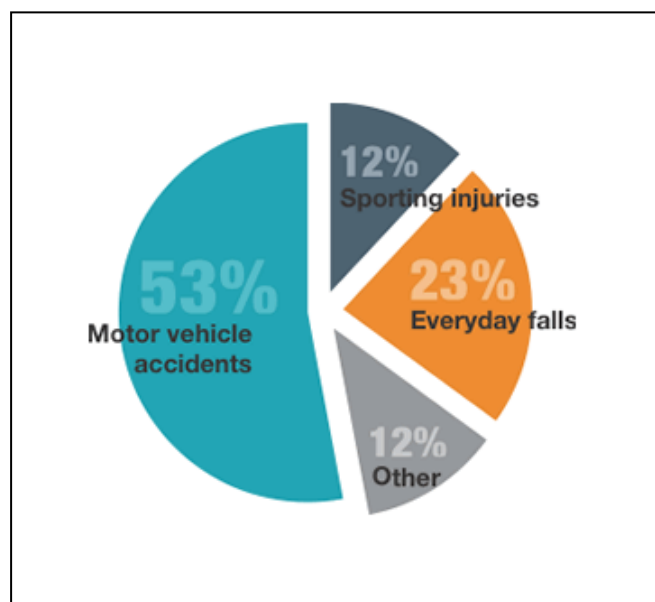


Figure 3. Causes of traumatic SCI

2.2 Outcomes and symptoms of SCI

Very often SCI leads to permanent paralysis and loss of sensibility below the lesion site, resulting in four main clinical pictures: paraplegia, quadriplegia, paraparesis, quadriparesis.

Paraplegia means a sensorimotor syndrome with lower limb paralysis and sphincter disturbances, while in quadriplegia are also added upper limb paralysis and neurodegenerative disorders; paraparesis and quadriparesis are respectively incomplete paralysis of the lower limbs and incomplete paralysis of four limbs.

The degree of disability after SCI depends primarily on two factors: the level of neurological lesion and the completeness of the lesion.

The neurological level of injury refers to the segment of the spinal cord caudal to the injury which preserves normal motor and sensory functions on both sides of the cord. If a patient, for example, has a normal sensibility at the C3 level, but the absence of sensibility at level C4, the neurological level is at C3.

SCI can be classified as complete or partial: complete lesion describes a total loss, and usually permanent, of the ability to send sensory nerve impulses and start a movement and a complete loss of function below the level of the lesion. This causes complete paraplegia or quadriplegia. Partial SCI refers instead to partial spinal cord damage: in this situation some motor and sensory functions are maintained.

The main symptoms resulting from a partial or complete SCI are:

- Loss of motor functions. In addition to paralysis, people with SCI have muscle tone changes: spasticity can cause spontaneous contractions, while the flaccidity makes the muscles weak and soft, without muscle tone.
- Loss of sensitivity, including the ability to feel heat, cold and touch.

Below the neurological level of the lesion, there is the loss of sensitivity of a part or all the skin.

- Loss of bladder control that increases the risk of urinary tract infections and it can even cause urinary infections or the formation of kidney or bladder stones.
- Loss of bowel control. The nervous system is no longer able to control the muscles that open and close the anus, causing fecal incontinence.
- Exaggerated reflex activity or spasms.
- Disorders of sexual activity and fertility.
- Pain or a sensation of intense burning caused by damage to the nerve fibers in the spinal cord; allodynia is a type of neuropathic pain where a higher sensitivity to normally harmless stimuli is manifested.
- Difficulty in breathing, coughing, or lungs secretion. There is also frequent involvement of abdominal muscles and chest. The neurological level of the lesion determines which type of respiratory problem the patient presents. Cervical and thoracic lesions present greater risks.
- Circulatory problems. Injury increases the risk of blood clots, such as deep venous thrombi or lung emboli and, in some cases, can cause a permanent increase in blood pressure (autonomous hyperreflexia).

The most common tool used by physicians to classify the neurological deficit and the extent of the injury is the ASIA scale, initially developed by Spinal Injury Association (ASIA) (Fig. 5).

The ASIA classification is based on a careful assessment which maps any sensory and motor function and includes five degrees that describe the severity of SCI:

A = Complete: no motor-sensory function is preserved (below the level of injury, including the sacral segments S4-S5).

B = Incomplete: the sensory function, but not the motor is kept below the level of injury (including the sacral segments S4-S5).

C = Incomplete: motor function is preserved below the level of injury, and more than half of key muscles below this level have a muscle degree less 3.

D = Incomplete: motor function is preserved below the level of injury, and at least half of the key muscles below this level have a muscle degree of 3 or more.

E = Normal: motor and sensory functions are normal.

The evaluation should be performed 72 hours after the lesion, as at this time prognostic values seem to be more accurate than those obtained in shorter times (Herbison and Theodosios, 1991).

Patient Name _____
 Examiner Name _____ Date/Time of Exam _____

ASIA
 AMERICAN SPINAL INJURY ASSOCIATION

STANDARD NEUROLOGICAL CLASSIFICATION OF SPINAL CORD INJURY **ISCS**

MOTOR
 KEY MUSCLES (scoring on reverse side)

	R	L		R	L
C5			Elbow flexors		
C6			Wrist extensors		
C7			Elbow extensors		
C8			Finger flexors (distal phalanx of middle finger)		
T1			Finger abductors (distal finger)		
UPPER LIMB TOTAL (MAXIMUM) (25) (25) (50)					
Comments					
L2			Hip flexors		
L3			Knee extensors		
L4			Ankle dorsiflexors		
L5			Long toe extensors		
S1			Ankle plantar flexors		
Lower limb TOTAL (MAXIMUM) (25) (25) (50)					
TOTALS (MAXIMUM) (50) (50) (100)					

SENSORY
 KEY SENSORY POINTS

0 = absent
 1 = impaired
 2 = normal
 NT = not testable

Any anal sensation (Yes/No) _____
 PIN PRICK SCORE (max: 112) _____
 LIGHT TOUCH SCORE (max: 112) _____

NEUROLOGICAL LEVEL
 The most caudal segment with normal function.

COMPLETE OR INCOMPLETE?
 Incomplete = Any sensory or motor function in S4-S5

ASIA IMPAIRMENT SCALE

CLINICAL SYNDROMES (OPTIONAL)

☐ Central Cord
☐ Brown-Sequard
☐ Anterior Cord
☐ Conus Medullaris
☐ Cauda Equina

REV 03/06

MUSCLE GRADING

- 0 total paralysis
- 1 palpable or visible contraction
- 2 active movement, full range of motion, gravity eliminated
- 3 active movement, full range of motion, against gravity
- 4 active movement, full range of motion, against gravity and provides some resistance
- 5 active movement, full range of motion, against gravity and provides normal resistance
- 5* muscle able to exert, in examiner's judgement, sufficient resistance to be considered normal if identifiable inhibiting factors were not present

NT not testable. Patient unable to reliably exert effort or muscle unavailable for testing due to factors such as immobilization, pain on effort or contracture.

ASIA IMPAIRMENT SCALE

- ☐ **A = Complete:** No motor or sensory function is preserved in the sacral segments S4-S5.
- ☐ **B = Incomplete:** Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5.
- ☐ **C = Incomplete:** Motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3.
- ☐ **D = Incomplete:** Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade of 3 or more.
- ☐ **E = Normal:** Motor and sensory function are normal.

CLINICAL SYNDROMES (OPTIONAL)

- ☐ Central Cord
☐ Brown-Sequard
☐ Anterior Cord
☐ Conus Medullaris
☐ Cauda Equina

STEPS IN CLASSIFICATION

The following order is recommended in determining the classification of individuals with SCI.

- Determine sensory levels for right and left sides.
- Determine motor levels for right and left sides.
Note: in regions where there is no myotome to test, the motor level is presumed to be the same as the sensory level.
- Determine the single neurological level.
This is the lowest segment where motor and sensory function is normal on both sides, and is the most cephalad of the sensory and motor levels determined in steps 1 and 2.
- Determine whether the injury is Complete or Incomplete (sacral sparing).
If voluntary anal contraction = No AND all S4-5 sensory scores = 0 AND any anal sensation = No, then injury is COMPLETE. Otherwise injury is incomplete.
- Determine ASIA Impairment Scale (AIS) Grade:
Is injury Complete?
 NO → **Is injury motor incomplete?**
 YES → **Are at least half of the key muscles below the (single) neurological level graded 3 or better?**
 NO → AIS=C
 YES → AIS=D
 If YES, AIS=A Record ZPP (For ZPP record lowest dermatome or myotome on each side with some (non-zero score) preservation)
 If NO, AIS=B (Yes=voluntary anal contraction OR motor function more than three levels below the motor level on a given side.)

If sensation and motor function is normal in all segments, AIS=E
Note: AIS E is used in follow up testing when an individual with a documented SCI has recovered normal function. If at initial testing no deficits are found, the individual is neurologically intact; the ASIA Impairment Scale does not apply.

Figure 4. ASIA scale

3. SCI IN EXPERIMENTAL ANIMAL MODELS

Animal models offer the possibility to study the pathophysiological mechanisms of SCI and to test *in vivo* experimental therapeutic strategies, a fundamental step that precedes clinic application. Up to now, the use of animals is the only preclinical approach able to have a translational potential to the clinic.

Rodents are by far the most widely used animal model as they have a number of clear advantages: reproducibility of the disease, low cost, easy to care for and ability to conduct large number studies, post-surgical infections are rare. Well-established functional analysis methods, rodents are moderate size that allows one to easily perform behavioral and histological tests.

In experimental animal models, three general classes of injury are frequently used: **transection**, **contusion** and **compression** (Rosenzweig and McDonald, 2004).

Transection is induced by cutting the spinal cord following laminectomy. Because all the nerve fibers are severed, this model offers the advantage of easier evaluation of the therapeutic efficacy of treatments that promote axonal regeneration. The main disadvantages are that it reproduces a type of injury, complete transection, of rare clinical occurrence and, above all, the post-surgical care of transected animals is very difficult.

Incomplete transection (e.g. **hemisection**) is used for the evaluation of treatments aimed to re-grow and regenerate spared axons and for evaluating the correlation between anatomical changes and specific behavioral improvements. Hemisection preserves the integrity and function of one side of the cord, which reduces the need for postoperative care and provides an internal control on the side of the cord not damaged. At the same time, it makes it rather difficult to distinguish the benefits resulting from the therapeutic procedure being tested and those due instead to physiological reorganization (Zorner et al.; 2010). However, incomplete transection such as those produced by hemisection are rarely seen in human patients, so many researchers prefer to use compression and contusion injury models.

The **contusion** and **compression** models present a neuropathological course and histological characteristics that reproduce those present in humans following trauma. In fact they trigger a series of biochemical processes that lead to a cell necrosis confined primarily within the SC, which correlates with the loss of mobility.

Contusion is induced by hitting the exposed spinal cord with a blunt contusion force. The first contusion animal model was proposed in 1911 by Allen: it was a weight dropped on the spinal cord of a dog previously submitted to laminectomy (Anderson, 1982).

Subsequently, a wide range of methods have been developed over the years to induce injury into the SC via external contusion, in order to optimize the intensity, the duration and the accuracy of biomechanical

forces applied to obtain a reproducible lesion. Now this model is often obtained using an impactor, which consists of a device that delivers a weight (New York University impactor, Gruner et al.; 1992) or a solenoid (Ohio State University impactor, Behrmann et al.; 1992) to the SC, with a computer monitoring the impact.

A recent computer-controlled contusion device is the Infinite Horizon (IH), which uses force instead of tissue displacement and is the first able to inflict reproducible injuries without touching the SC before the hit.

The **compression** model was introduced by Rivlin and Tator in 1978, inducing injury by compressing the SC with a modified aneurysm clip (Rivlin and Tator, 1978).

The compression model can create different degrees of SCI adjusting the compression strength and/or time. This model also mimics the human neuropathology. It begins with a spreading of hemorrhagic necrosis and edema, progressing to a phase of partial repair and reorganization up to a chronic phase with axonal atrophy and the formation of a glial scar. The clip compression model has provided valuable knowledge about the pathophysiology of SCI (Fehlings et al.; 1989), and neuroprotective agents (Schwartz and Fehlings, 2001).

3.1 Assessment of recovery

Behavioral analysis (motor and sensory), histological staining and electrophysiology are the most used techniques to evaluate functional and anatomical recovery.

Behavioral evaluation: The most commonly used test to assess motor function after SCI is the Basso Mouse Scale (BMS) for mice or the Basso, Beattie and Bresnahan (BBB) locomotor scale for rats. Note that the locomotor recovery indices are not comparable between the two models. BMS consists of a 9-point scale divided into 3 main phases of motor recovery: an early phase based on ankle movement (0-2), an intermediate phase with plantar placing and stepping (3-5) and a late phase of recovery based on fine movements details (coordination, paw placement, stability, 5-9) (Basso et al.; 2006).

BBB locomotor scale is a 21-point scale where 0 corresponds to no apparent movement and 21 represents a normal coordinated movement. Scores from 0 to 7 are related with recovery of isolated movements in the hip, knee and ankle. Scores from 8 to 16 are associated with plantar placement and coordination of forelimbs; from 14 to 21 are evaluated paw and tail position, toe clearance and trunk stability (Basso et al.; 1995).

In order to decrease the subjectivity of both tests, they should be performed by two independent researchers.

Other tests able to evaluate the locomotor recovery after SCI are:

CatWalk: computer-assisted gait analysis useful to measure different dynamic parameters such as limb base of support, stride length, velocity, interlimb coordination (Hamers et al.; 2001). The major limitation is that animals must be capable of at least occasional plantar stepping.

Horizontal ladder walking test: in this test the animal walks along a horizontal ladder with variable rung spacing. The frequency of mis-steps is calculated as the number of foot slips by each limb divided by the total number of steps. Also this test cannot be used in complete paralyzed animals (Metz and Whishaw, 2002).

3D Analysis of musculoskeletal movement: with the use of several video cameras and reflective markers attached to the animal skin, it is possible to obtain a precise geometric locomotor profile of the animal and to detect fine motor improvements. Some disadvantages of this technique are costs, time and the lack of standardization (Zorner et al.; 2010).

The assessment of *sensory function* in rodents is usually performed with the **hot plate test** and **Von Frey filaments test**.

The first evaluates the hind paw withdrawal latency from a radiant heat source, which is increased in the case of thermal hyperalgesia after SCI (altered perception of temperature) (Woolf and Thompson, 1991).

The second test is used to evaluate the mechanical allodynia (increased sensitivity to innocuous stimuli caused by neuropathic pain). Filaments

are sequentially applied to the plantar surface of the limbs, with a pressure that causes a slight bend of the filament, until the withdrawal response is obtained. A positive reaction occurs when the paw is quickly removed away from the filament. This response may be associated with vocalization, flinching and/or abnormal aggressive behavior (Gris et al., 2004). It is important to note that sensory tests are usually difficult to interpret as they virtually all depend on a motor end point that could, of course, be compromised by SCI.

Anatomical analyses: immunohistochemistry provides knowledge about anatomic changes, pathophysiology and axonal regeneration. Specific markers are associated to specific hypothesis to be tested. Examples of biomarkers associated to SCI-related experiments are summarized in table 2.

Marker	Information about	References
NF	Axonal organization	Kaneko 2006
CD11b/OX42	Microglia	Detloff 2008
GFAP	Astrocytes	Liu 2010
APC	Mature Oligodendrocytes	Karimi-Abdolrezaee 2006
MBP	Myelin sheath	Woerly 2001
5HT	Serotonergic Neurons	Kaneko 2006

Table 2. Most popular biomarkers employed in SCI experiments (adapted from Silva et al.; 2014)

Another helpful tool for identifying cellular populations involved in inflammation or regenerative mechanisms are transgenic mice expressing fluorescent labeling in specific CNS cells, such as neurons, microglia, and astrocytes. Indeed, the most definitive way of verifying axon regeneration is to document the process while it is happening. Live images of an axon re-growing from its injured tip could be considered as possible evidence of axon regeneration. *In vivo* imaging of single axons in the spinal cord requires animal models with appropriate fluorescent labeling. A widely used transgenic mouse line has been developed by Feng *et al.*, in which different fluorescent markers are expressed under the Thy1 promoter in specific subsets of neurons. Using the GFP-S line of these transgenic mice, Kerschensteiner *et al.* described the response of lesioned ascending dorsal sensory axons through repetitive imaging *in vivo* (Kerschensteiner et al.; 2008). Again, by using CX3CR1-GFP mice expressing Enhanced Green Fluorescent Protein (EGFP) in brain and SC microglia under control of the endogenous Cx3cr1 locus, it is possible to evaluate the inflammatory response after SCI.

4. PATHOPHYSIOLOGY OF SCI

The pathological events following acute SCI are divided into two broad chronological events: the **primary injury** and the **secondary injury**, due to the additional damaging processes initiated by the primary injury (Taoka et al.; 1998; Dumont et al.; 2001; Priestley et al.; 2012).

The pathophysiological mechanism of SCI is more than a simple mechanical disruption of nerve transmission following injury. In addition to the primary lesion of the spinal cord, a multi-step cascade results in progressive enlargement of the injury, due to factors that include an inflammatory reaction, ischaemia, oedema, haemorrhage and cytotoxicity (Ronsyn et al.; 2008). Although there is little or no loss of interneurons and motor neurons in several segments beyond the injury, there are significant changes in their biochemical, and consequently physiological, properties (Petruska et al.; 2007; Button et al.; 2008; Roy et al.; 2012). Various studies indicate that there are strong correlations between human and rodent spinal injuries (Metz et al.; 2000; Cheriyan et al.; 2014). The similarities between the human and rodent inflammatory response (Fleming et al.; 2006) and the morphological response (Norenberg et al.; 2004) after SCI permit useful translation to clinical use of treatments developed in animals. Experimental rat SCI models are also similar to the human SCI with respect to motor function recovery related to electrophysiological changes, such as motor evoked potentials and somatosensory evoked

potentials, and lesion size assessed using MRI examination (Metz et al.; 2000). Thus, it is essential to use reliable animal models to assess the pathophysiological mechanisms of SCI and to investigate neuroplasticity in the intact spinal cord caudal to the lesion site.

4.1 Primary injury

The primary injury is represented by an immediate mechanical damage to the SC (contusion, compression, or laceration) that leads to a hemorrhagic zone of necrosis in the grey matter. Neurons and their axons become permeabilized acutely following injury due to compressive and shear forces. Animal studies have demonstrated that neurological impairment increases relative to the force of trauma and the duration of compression. Clinically, there are several types of primary injury. The most common mechanism involves impact with persistent compression (Tator, 1996). This happens in burst fracture with bone fragment compression of the SC, fracture-dislocation or disc rupture following injury. Another mechanism of primary injury is caused by flexion, extension, rotation or dislocation, producing shearing or stretching of the SC. This type of injury may be present without obvious radiological evidence of trauma, but it is common in adults with underlying degenerative spine disease (Tator, 1996). Sharp knife cutting and severe distraction leads to laceration and transection injury, which is another type of mechanism. This type of injury may occur to

varying degrees in SCI, from minor injury to complete transection. The primary mechanical injury is the starting point for additional secondary mechanisms of injury extend. Consequently, the damage can spread from the lesion epicenter to caudal and rostral segments.

4.2 Secondary injury

The mechanical injury leads to a cascade of neuropathological events and the secondary phase of the injury begins. This response of SCI can be divided into three phases, an acute phase, subacute phase and a chronic phase (Tator, 1995; Bareyre et al.; 2003).

The **acute phase** represents the first 48 hours after injury and is a direct result of physical trauma to the nervous tissue that causes death of cells near the site of injury. The main cells involved are neurons, astrocytes, oligodendrocytes and endothelial cells. The death of the endothelial cells of local blood vessels causes bleeding, which alters the supply of oxygen and nutrients to the tissue, causing additional necrotic cell death. During the acute phase of the injury the body responds by activating the complement cascade and inducing a massive infiltration of neutrophils into the damaged site and in the adjacent parenchyma (Donnelly and Popovich, 2008). As a consequence, there is edema at the site of the SC lesion. The hemorrhagic area, initially confined to the gray level of the substance which is very vascularized, extends at a

later time (from minutes to hours) at the level of the white matter in the rostro-caudal direction. This event continues in the sub-acute phase and sometimes even for months during the chronic phase.

The **subacute phase** develops within minutes and can last several weeks following injury, up to 6 months in humans. During this phase we are witnessing a worsening of the initial damage mainly due to the local inflammatory response. In addition, the cell death due to trauma increases the levels of amino acids, such as glutamate, the excitotoxicity contributing extra-cellular fluid, while high levels of calcium activate enzymes that damage cellular structures (phospholipase, protease). The microglial cells are activated and remain in this state for up to four months after injury. The lipid peroxidation and the formation of free radicals contribute to the necrosis of the tissue. There is also the infiltration of neutrophils (6-24 hours), macrophages (24 hours – 2 weeks) and T lymphocytes in the damaged tissue during the first week after injury (Bethea et al.; 2002). Oligodendrocytes and vulnerable neurons undergo apoptosis (Liu et al.; 1997).

The **chronic phase**, which persists throughout life, is characterized by the stabilization of the lesion through the formation of the scar that has the function of confining and separating the lesion from the damaged tissue. However, at the same time this prevents the regeneration of nerves. The scar is surrounded by fibroblasts, activated macrophages and glial cells and often surrounds a cyst or cavity (Bruce et al., 2000).

A progressive expansion of the lesion in more than one segment, a process called syringomyelia, can occur for months or years after the lesion, increasing the severity of the lesion and causing death in some cases.

5. SPONTANEOUS FUNCTIONAL RECOVERY AFTER SCI

The neuroplasticity of the CNS allows some degree of functional recovery after SCI. The process of learning, skill acquisition and in particular the response to an injury leads to neuronal reorganization, synaptic rearrangements, changes in neuronal activation pattern and axon collateral sprouting. Many studies show that functional recovery occurs in both humans and rodents, particularly after incomplete injuries (Weidner et al.; 2001; Tetzlaff et al.; 2009). This finding indicates that reorganization throughout the neuraxis can compensate for the disrupted pathways and rebuild a circuit for control of motion. Although this neuroplasticity has been related to spontaneous functional recovery (Onifer et al.; 2011), the underlying mechanisms are still largely unknown.

5.1 Neuroplasticity following SCI

Following injury, neurons are capable of mounting a transient regenerative response, as evidenced by the expression of regeneration

associated proteins and genes (Kruse et al.; 2011). The most commonly upregulated genes and transcription factors that are found to be associated with regeneration are: c-jun, activating transcription factor 3 (ATF-3), heat shock protein 27 (HSP-27), growth associated protein-43 (Gap-43), signal transducer and activator of transcription-3 (STAT3) (Sun et al.; 2010). Again the upregulation of molecules that contribute to axonal regrowth, such as neuronal calcium sensor-1 (NCS-1) (Yip et al.; 2010) and mammalian target of rapamycin (mTOR) (Liu et al.; 2010), can induce compensatory sprouting from unlesioned nerve pathways. Furthermore, a number of the molecules that can guide growing axons in the developing nervous system are also present in the injured spinal cord (Jacobi et al.; 2014). These factors can support growing collaterals and initiate synaptogenesis during circuit remodeling. This indicates that the intrinsic growth program inherent to neurons is activated in response to injury; however, it is not sustained.

5.2 Reorganization of descending pathways after SCI

Recently, it has been recognized that spontaneous and treatment-induced functional recovery is correlated with spared descending axons and myelin (Fouad et al.; 2001; Weidner et al.; 2001). It has been demonstrated by using electrophysiology and retrograde neurotracing (Bareyre et al.; 2004), that the new neuronal circuits make functional connections. Adult neurons in the CNS cannot regenerate over long distances, but axotomized neurons can form short sprouts from their

damaged axons (Fawcett et al.; 1998). The injury provides a stimulus for intact interneurons, white matter tracts and sensory neurons to replace inputs to the spinal neurons that have lost synapses. Collateral or regenerative sprouting of intraspinal axons after SCI may provide an opportunity to make new connections or to strengthen existing synapses on denervated spinal neurons (Brown et al.; 2012). A probable mechanism that leads to collateral formation is the upregulation of diffusible attracting factors by the denervated tissue. One study showed that the reorganization of corticospinal tract (CST) circuits is guided by brain derived neurotrophic factor (BDNF) secretion by the target interneurons. Furthermore, knockdown of BDNF in spinal neurons diminished the formation of new CST pathways (Ueno et al.; 2012).

5.3 Modulation of the synaptic transmission

There are three different mechanisms able to alter neural activity: (1) modifying synaptic transmission at pre-existing synapses, (2) eliciting the growth of new synaptic connections or the pruning away of existing ones, or (3) modulating the excitability properties of individual neurons (Crupi et al.; 2013). SCI denervates spinal neurons by disrupting ascending and descending pathways to and from the brain as well as output to lower motor neurons. The loss of part or all synaptic inputs results in structural modifications in the number, size, and distribution

of synaptic contacts on interneurons and motor neurons (Raineteau et al.; 2001). One way that synaptic changes after SCI have been detected is by comparing the synaptophysin level in intact versus lesioned spinal tissues. (Nacimient et al.; 1995). Synaptophysin is a protein within synaptic vesicles in the presynaptic terminal. The expression of synaptophysin is decreased after a spinal cord lesion, but the level recovers to normal after a few weeks. This finding suggests that new synapses form on motor neurons following SCI, and this formation could arise from interneurons or sensory afferents transmitting tactile and proprioceptive signals. The mechanism with strongest evidence in mediating changes within neuronal networks is the removal of GABAergic inhibition in excitatory synapses. Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the CNS and plays a "counter balance" role against enhanced synaptic transmission as a result of glutamate-mediated excitation of neurons following SCI. GABA has a presynaptic inhibitory action on primary afferents on postsynaptic membranes of interneurons and motor neurons (Alvarez et al.; 1996). After complete SCI, Roy and collaborators demonstrated that the GABAergic intracortical inhibitory circuit is reduced, resulting in altered inhibition during the post-lesion period (Roy et al.; 2011). These changes in synaptic efficiency and neurotransmission may be important during the period of post-lesion recovery and addressing these changes may be important for a successful therapy after SCI.

5.4 Reorganization of cortical areas

Following traumatic SCI, axonal architecture is disturbed and the brain grey matter becomes atrophic (Jurkiewicz et al.; 2006; Freund et al.; 2011; Henderson et al.; 2011; Rao et al.; 2013). Furthermore, in response to SCI, the sensorimotor cortex can undergo a dramatic reorganization. Remodeling of the S1 map correlates with tactile discrimination performance (Xerri et al.; 2005; Rao et al.; 2013), while different populations of cortical neurons in M1 cortex selectively contribute to the execution and regulation of movements during locomotion in cats (Drew et al.; 2002). This cortical reorganization is sometimes found to be compatible with functional recovery (Schmidlin et al.; 2004; Rao et al.; 2013). It was previously described that cortical territories controlling intact body parts tend to enlarge and invade cortical areas that have lost their peripheral targets (Qi et al.; 2000). Although the underlying mechanism of cortical reorganization is not well clarified, some studies have suggested that these events are partly due to changes in neuronal circuits, such as axonal sprouting (Bareyre et al.; 2004; Schmidlin et al.; 2004; Martinez et al.; 2010).

6. OVERCOMING THE LIMITED REGENERATION OF THE CENTRAL NERVOUS SYSTEM

Unlike the peripheral nervous system (PNS), in which axons are able to regenerate after a lesion, in the CNS there is an almost total absence of nerve regeneration. In the literature it has been shown that neurons from PNS, able to regenerate in their district of residence, are not able to do equally well in the CNS lesion site level, while neurons from the CNS are able to regenerate at the lesion site in the PNS (Chong et al.; 1996; Yiu and He; 2006), suggesting that an enabling environment is necessary for axon regeneration.

In particular, the causes of this deficit appear to be:

- an **inhibitory external environment**, characterized by the presence of inhibitory molecules produced by myelin and the glial scar;
- a **repression of intrinsic regenerative pathways** (neurons), which is due to an adjustment of the RAG (Regeneration Associated Genes) and a lack of trophic factor production.

6.1 Myelin Inhibition

A specific inhibition of CNS regeneration has been related mainly to white matter rather than to grey matter (Savio et al.; 1989) and, in particular, to myelin associated-inhibitors (**MAIs**). The expression of

specific inhibitory proteins causes cytoskeletal rearrangements leading to growth cone collapse and inhibition of neurite growth.

The first MAI discovered was the Myelin-associated glycoprotein (**MAG**) in 1994, from two different groups (McKerracher et al.; 1994; Mukhopadhyay et al.; 1994). However, myelin derived from MAG-deficient mice is already inhibitory for axonal regeneration (Ng et al.; 1996; Bartsch et al.; 1995), suggesting that other inhibitory proteins are produced.

Other inhibitory proteins are produced by the Nogo gene (Chen et al.; 2000). It encodes for three different splice variants: Nogo-A that is the inhibitory molecule expressed by oligodendrocytes (the long isoform), Nogo-B (intermediate isoform) and Nogo-C (short isoform) that are expressed outside the CNS. A conserved domain present in all the Nogo proteins is Nogo-66, a C-terminal domain exposed to the extracellular space that possesses the inhibitory effect on axonal regeneration (GrandPre et al.; 2000). Nogo is expressed only by oligodendrocytes, not by Schwann cells, and this could, at least in part, explain the different permissive environment for axonal regeneration between CNS and PNS. Experiments in mice lacking one of three Nogo isoforms led to inconsistent results (Simonen et al.; 2003; Zheng et al.; 2003; Kim et al.; 2003), maybe due to the compensatory effect by MAG and other MAIs. Another confounding factor could be also the differential

expression of Nogo A levels by mice having a different genetic background (Dimou et al.; 2006).

Oligodendrocyte myelin glycoprotein (OMgp) is the third MAI identified and expressed on CNS myelin (Wang et al.; 2002; Kottis et al.; 2002). OMgp is a GPI-anchored CNS myelin protein expressed not only by oligodendrocytes but also by neurons (Habib et al.; 1998). After Knocking down OMgp, a less inhibitory environment is obtained, although with no improvement of axonal sprouting or motor function (Ji et al.; 2008).

All MAIs mentioned above (MAG, Nogo66 and OMgp) act on a single neuronal receptor, called Nogo receptor (NgR1) (Fournier et al.; 2002; Domeniconi et al.; 2002; Wang et al.; 2002; Borrie et al.; 2012). These MAIs bind to the same NgR1 domain (N-terminal leucine-rich repeat (LRR) domain), indicating a redundant ligand model. Since NgR is a GPI-linked protein, it lacks the intracellular domain necessary to transduce signals inside the cells. Thus, efforts have been made to identify co-receptors of NgR. The first NgR co-receptor identified is p75, which mediates the downstream signaling of all three MAIs (Yamashita et al.; 2002; Wang et al.; 2002). The pre-incubation of neurons with neurotrophins could prevent the inhibition mediated by MAIs, but, after injury, they could not interfere anymore (Cai et al.; 1999). Results of knock-out mice for NgR are inconsistent and controversial regarding the axonal regeneration (Zheng et al.; 2005; Kim et al.; 2004) maybe

suggesting the involvement of other receptors for the MAIs action on regeneration inhibition. Once activated, NgR co-receptors transmit the intracellular signal through RhoA, a small GTPase protein which activates ROCK (Rho-A associated kinase) and PKC (Protein kinase C). These are regulators of the actin cytoskeleton and cause a stiffening of the cytoskeleton leading to an inhibition of the axons elongation and to a growth cone collapse, as is well explained by Yiu review (Yiu et al.; 2006).

MAIs are not the only inhibitors of neurite growth. Other proteins that could be both chemorepellants and chemoattractants are Semaphorin family (Moreau-Fauvarque et al.; 2003), Ephrins (Benson et al.; 2005), Netrins (expressed by oligodendrocytes, cells of the central canal and the meninges) (Culotti et al.; 1996), Repulsive guidance molecule a (RGMa) (Schwab et al.; 2005), Wnt proteins (expressed during development to guide axons and upregulated in adult only after injury) (Liu et al.; 2008) and Lynx1 (Morishita et al.; 2010).

In order to counteract the axon's regeneration inhibition by MAIs, different approaches have been tested. In general, we can distinguish two main strategies: I) blocking the recognition between inhibitory ligands and their receptors, II) blocking the intracellular common pathways.

Regarding the first approach, the most studied is the counteracting of Nogo-A ligands. Indeed, the use of drugs anti-Nogo is the longest

studied approach for SCI (Kwon et al.; 2011) that started in 1990 with the IN-1 monoclonal antibody against Nogo-A, reviewed by (Buchli et al.; 2005). This approach was able to enhance regeneration and compensatory sprouting, together with functional improvement. Also the humanized anti-Nogo antibody (ATI-355) is able to enhance sprouting and functional recovery in primates (Freund et al.; 2006).

Other approaches have been used on soluble receptors of NgR (Fournier et al.; 2002) which demonstrated functional improvements (Li et al.; 2004) also with a 3 day delay from the primary injury (Wang et al.; 2006). Moreover, recently, a lentiviral mediated expression of a soluble NgR in the intracellular space has been achieved, leading to an increased axon regeneration (Zhang et al.; 2013).

Regarding the other inhibitory molecules produced by myelin debris, different strategies have been used to block Semaphorins. These include Sema3A antagonists (SM-216289), which show some results in regeneration and functional improvements (Yamashita et al.; 2003), anti-Sema3A peptide (Montolio et al.; 2009) and Xanthofulvin (SM-216289) to inhibit Sema3A, which promotes regeneration and functional recovery (Kaneko et al.; 2006). An anti EphA4 peptide has been also utilized and the authors reported increased CST sprouting and an improved recovery (Fabes et al.; 2007).

The second strategy aims to inhibit more than one MAI at the same time, acting on common intracellular pathways. Different inhibitors have

been used, such as ROCK inhibitors, (Fournier et al.; 2003), RhoA inhibitors (Dillon et al.; 1995) or PKC inhibitors (Sivasankaran et al.; 2004). However, negative effects associated with Rho/ROCK inhibition have also been reported. Although McKerracher's group documented corticospinal tract regeneration in mice (Dergham et al.; 2002) and neuroprotection in both rat and mice (Dubreuil et al.; 2003) with acute C3 treatment, others have found increased spinal cord tissue atrophy (Fournier et al.; 2003) and detrimental health effects (Sung et al.; 2003) in rats. Again, for Y27632, certain dosages of this drug actually decreased axonal sprouting/regeneration, and impaired functional recovery (Chan et al.; 2005, Sung et al.; 2003).

Another strategy to block the inhibition of axonal regeneration is to modify the cAMP levels. Indeed, it has been demonstrated that some myelin associated proteins are able to decrease cAMP neuronal levels limiting the axonal re-growth (Hannila et al.; 2008). Hence, cAMP depletion is an important pharmacological target for a neuronal regeneration approach, because the increase of cAMP overcomes the inhibition by MAG and myelin. A promising drug able to act on the cAMP levels is Rolipram. This drug inhibits phosphodiesterase 4, thereby raising the level of cAMP.

It is now clearly demonstrated that the deletion of inhibitory proteins is able to enhance sprouting, but it does not affect motor behavior. This may suggest that these inhibitors are more involved in the sprouting

than in CNS regeneration failure (Lee et al.; 2010). Moreover, there is a redundant biochemical pathway both for inhibitory molecules produced by myelin and for inhibitory receptors present in neurons. For this reason, the deletion or the suppression of only one signal is not sufficient to promote plasticity and subsequent functional recovery after SCI.

6.2 Glial Scar

The initial insult causing spinal cord injury is followed by numerous barriers that counteract successful axon regeneration, including the development of a glial scar. Indeed, several days after SCI, astrocytes surrounding the injury site begin to show hypertrophy with morphological and molecular changes. They accumulate, extend and overlap their processes forming tight junctions that constitute the scar around the injury site (Reier et al.; 1983, Fawcett et al.; 1999, Silver et al.; 2004). The scar also contains oligodendrocytes and microglia. The glial scar has a role in re-establishing the physical and chemical integrity of the CNS and for closing the blood brain barrier, reducing infiltration from the periphery, minimizing infections and the spread of cellular damage (Reier et al.; 1983, Faulkner et al.; 2004, Sofroniew et al.; 2009).

On the other hand, the glial scar produces chemical signals inhibiting axonal sprouting. In fact, between the factors involved in the lack of repair after SCI, glial scar-associated inhibitors such as tenascin, Semaphorin 3, keratin and chondroitin sulfate proteoglycans (KSPGs, CSPGs) play an important role (Silver et al.; 2004, Carulli et al., 2005). CSPGs are extracellular matrix molecules consisting of a protein core with one or more covalently attached chondroitin sulphate glycosaminoglycan (CS-GAG) chain. Chondroitin sulphate readily interacts with proteins in the extracellular matrix due to its negative charges and these interactions are important for regulating a diverse array of cellular activities, such as pathfinding and guidance during development and limitation of neuronal plasticity in the adult CNS (Corvetti et al.; 2005; Fukuda et al.; 1997). The CSPG family includes aggrecan, neurocan, versican, brevican, NG2 and phosphacan/DSD-1. Following SCI, CSPGs are upregulated around injury sites (Asher et al.; 2000, 2002; Jones et al.; 2003; Tang et al.; 2003) and it has been demonstrated that this upregulation can inhibit neurite outgrowth in vitro (Snow et al.; 1990; Smith-Thomas et al.; 1994, 1995; Dou and Levine, 1994, 1997) and in vivo (Davies et al.; 1997). Therefore, its degradation has become a key therapeutic goal in the field of CNS regeneration. Evidence that the GAG sugar chains are a major inhibitory component of CSPG molecules have come from studies which have utilized the bacterial enzyme chondroitinase ABC (ChABC), that is able to digest chondroitin sulfate glycosaminoglycans (CS-GAGs) of CSPGs.

There is robust pre-clinical evidence demonstrating beneficial effects of ChABC treatment following SCI. Furthermore, these effects have been replicated in a number of different injury models providing an important validation of ChABC as a promising therapeutic strategy. In particular, it has been demonstrated that *in vivo* ChABC treatment can render inhibitory substrates more permissive to growth (McKeon et al.; 1995; Zuo et al.; 1998; Yu and Bellamkonda, 2001; Grimpe et al.; 2005) and enhance axon regeneration after injury (Moon et al.; 2001; Bradbury et al.; 2002; Yick et al.; 2003, 2004; Chau et al.; 2004; Fouad et al.; 2005; Ikegami et al.; 2005; Houle et al.; 2006).

Mechanisms underlying these effects have been investigated. ChABC-mediated repair occurs partly through the digestion of the CS-GAG side chains, which are able to interact with PTP σ , a neuronal transmembrane receptor that mediates inhibition of axonal growth after interaction with CSPGs (Shen et al.; 2009, Fry et al.; 2010). Moreover, CSPGs are able to activate calcium dependent pathways, perturbing growth inhibitory responses in neurons (Sivasankaran et al.; 2004, Koprivica et al.; 2005, Monnier et al.; 2003).

In addition, CSPGs may hinder the growth-promoting potential of molecules expressed in the glial scar (such as laminin). Consequently, degrading CSPGs with ChABC may unmask this potential and allow growth to occur (Zuo et al.; 1998).

According to these studies, the abundant *de novo* synthesis of CSPGs in response to injury has also led to the hypothesis that CSPGs play also a

beneficial role in the repair process. In their works, A. Rolls et al. argued that CSPGs can control spatially and temporally the activity of infiltrating blood-borne monocytes and resident microglia, distinguishing between the beneficial role of CSPGs during the acute stage and its deleterious effect at later stages (Rolls et al.; 2008, 2009). These observations arise from the need to retain the endogenous potential of this molecule in repair by controlling its levels at different stages of post-injury repair.

In addition to ChABC treatment, two works showed different approaches to inhibit glial scar formation in mice models of SCI. The first demonstrates that it is possible to inhibit glial scar formation and facilitate regeneration after SCI using bioactive three-dimensional nanostructures, displaying high densities of neuroactive epitopes on their surfaces (Tysseling-Mattiace et al.; 2008).

In addition, a recent work shows how conditional Sox9 ablation reduces CSPG levels and improves motor function following spinal cord injury in mice (Mckillop et al.; 2013), suggesting a new molecular and pharmacological target.

6.3 Regeneration associated proteins (RAGs)

Following SCI, one of the main factors involved in the absence of axonal regeneration is the lack of an appropriate cell body response in terms of activating a proper pro-survival gene expression. In fact, regeneration

in the adult CNS of mammals is unsuccessful as a result of the decreased intrinsic regenerative capacity of affected neurons (Silver et al.; 2004).

A great contribution to the understanding of the regenerative mechanisms after injury, comes from the observation of neurons projecting into the peripheral nervous system (PNS), which are able to regenerate their axons after insult, in contrast to those confined to the CNS. In particular, the analysis of the differential gene expression response to axotomy in PNS and CNS neurons reveals that CNS neurons fail to up-regulate the expression of regeneration-associated proteins (RAGs), due also to the lack of trophic factors and their receptors (Fernandes and Tezlaff; 2000, Goldberg and Barres; 2000).

A strategy to stimulate the intrinsic machinery for neuronal regeneration is to lesion the neuronal peripheral axons before performing the central lesion or just after the injury. These phenomena are known as pre-conditioning and conditioning, respectively (McQuarrie et al.; 1973, Richardson et al.; 1984). An interesting issue arises from the observation that a pre-conditioning stimulus is more efficient in activating regenerative pathways if compared to conditioning after CNS injury, because the composition of a pro-regenerative environment is achieved before injury and CNS neurons are able to activate regenerative pathways before the glial scar formation.

Moreover, it has been shown that neurons within the CNS can be conditioned. Indeed, a regeneration-enhancing effect has been stimulated in adult mammalian retinal ganglion cells by lens injury or by an inflammatory agent placed within the vitreous body (Yin et al.; 2003). Again, a peripheral conditioning lesion stimulates axonal regeneration after a CNS lesion, activating a retrograde signaling pathway, setting neurons in a growth-competent state within 2 days (Yudin et al.; 2008, Hanz et al.; 2003). Among the various effects of such conditioning lesions, the most important appears to be the renewed activation of RAGs, such as GAP-43 (Plunet et al.; 2002, Ylera et al.; 2009). GAP-43 is required for neurite growth since it regulates the actin cytoskeleton. Its expression in injured CNS neurons is only transient and it decreases depending on the distance from the lesion to the cellular body (Fernandes et al.; 1999). However, increasing the expression of only one RAGs, such as GAP-43, seems to be not sufficient to increase neuronal regeneration (Buffo et al.; 1997, Mason et al.; 2000). Probably, the simultaneous expression of other RAGs is necessary, such as c-jun, α -tubulin, CAP-43 or NCAM (Bomze et al.; 2001, Plunet et al.; 2002, Kwon et al.; 2011).

Since a pre-conditioning stimulus has no translational potential for clinical use. Many efforts have been tried in order to mimic the stimulated responses of such stimuli in the neuronal cell body. Trying to act directly on the expression of RAGs, one proposed strategy is the

exogenous application of trophic support (BDNF or NT-4/5) directly to the neuronal cell body or by genetically modified cell lines. Trophic factors, indeed, are able to stimulate the neuronal expression of a large number of RAGs. This approach and the use of trophic factors in the SCI is discussed further later in this thesis.

To activate neuron-intrinsic growth it has also been proposed to use a gene transfer approach. In particular, gene transfer of transcription factors such as CREB, ATF3 or STAT3 are able to enhance axonal growth, even though in vivo effects are limited to short distance sprouting around the lesion site. This kind of therapy and its limitation are presented and discussed in the work of Franz and collaborators (Franz et al.; 2012).

On this basis, future research is needed to develop less invasive ways of stimulating RAGs expression, and also to evaluate these techniques in combination with strategies such as cellular bridging and/or glial inhibition, to overcome the obstacles imposed by the inhibitory environment.

6.4 Growth factors

The toxic environment produced after a lesion in the SC is characterized by a lack of trophic factors that fail to promote the axonal survival and guide axonal regeneration. This represents a further difference

between CNS and PNS, where Schwann cells produce spatially and temporally defined trophic factors, necessary to achieve a biologically active concentration that chemotactically guides axons (McCall et al.; 2012).

In a SCI context, neurotrophic factors could be used to prolong survival of injured neurons, to promote regeneration, to improve axons sprouting, to drive axonal growth, passing through the lesion, to influence glial development and in general to modify the environment making it more permissive for axonal growth. Trophic factors can be released by grafted cells or cells genetically modified to release a greater amount of a specific trophic factor, or by osmotic pumps, that deliver the trophic factors systemically or in situ, or by other polymeric delivery systems such as collagens and hydrogels (Katz et al.; 2009; Rossi et al.; 2013).

However, when used in single dose administration, neurotrophic factors revealed limited improvements, due to the impossibility of maintaining in vivo a constant biological effect. Indeed, ideally, the biological effect should be maintained until the regenerative processes finish, thus requiring multiple administrations or invasive therapies (Peleshok et al.; 2006).

For these reasons, gene therapy has been used to increase the amount of factors available for neurons and glia. Two kinds of therapy have been proposed in this field: a direct application of viral vectors to the

spinal cord, able to target a specific cell population (in vivo delivery), or a neurotrophin delivery by scaffolds or infected stem cells, which can provide also a growth substrate for axons (ex vivo delivery) (Bo et al.; 2011).

However, one concern about the use of artificial substrates is that they could trigger, once implanted in the injured spinal cord, a further inflammation response (Ronsyn et al.; 2008). For this reason, the use of cellular transplantation could provide an alternative method for the release of a great amount of trophic factors in the site of injury. Indeed, even though the real mechanism of action of stem cells is not yet known, one hypothesis is that they act through the release of trophic factors. It has been proved that bone marrow derived MSC (BM-MSCs) are able to secrete a great amount of neurotrophic factors, including, BDNF, NGF, VEGF and HGF (Chen et al.; 2002, Lin et al.; 2013, Quertainmont et al.; 2012). The advantages associated with BM-MSCs are their low immunogenicity, the possibility to be autologously transplanted and their immunomodulatory properties (Mothe et al.; 2012). This cell type has been tested also in several completed or ongoing clinical trials, demonstrating their safety and tolerability in SCI patients (Pal et al.; 2009; Yoon et al.; 2007; Sahni et al.; 2010). Overall, they reported favorable effects of MSCs on SCI patients due to the release of trophic factors and the recruitment of protective cells (Mothe et al.; 2012). Again, fetal stem cells are thought to act primarily

through the release of trophic factors (Coumans et al.; 2001), rather than to the cells themselves (Ronsyn et al.; 2008). The neurotrophic factor release seems to play a key role in the efficacy of neural stem cells, as well, after a SCI (Lu et al.; 2003; Gu et al.; 2012; Hawryluk et al.; 2012).

Independently from the route of administration, different neurotrophic factors have been used to date in SCI, each having specific function and peculiarities (Rosner et al.; 2012).

Nerve growth factor (NGF) is a trophic factor able to promote axonal regeneration and sprouting, primarily of nociceptive sensory axons (Hollis et al.; 2011). Fibroblasts genetically modified to produce NGF and inserted into the lesion cavity promote axonal regeneration of sensory axons (Grill et al.; 1997; Nakahara et al.; 1996) and local motor axons. However, NGF has no effect on the CST, and consequently promotes no functional improvement (Tuszynski et al.; 1997). An important feature of this trophic factor is that activity has been obtained in a wide therapeutic window in acute and chronic SCI (Grill et al.; 1997). However, it must be taken into account that NGF could have also deleterious effects, such as the development of neuropathic pain and cell death (Beattie et al.; 2002).

Brain-derived neurotrophic factor (BDNF) is one of the most studied trophic factors for the therapy of SCI. It suppresses the apoptosis of neurons and oligodendrocytes (Uchida et al.; 2012) and it is able to

reduce the necrotic zone and to promote neuronal survival (Novikova et al.; 1996; Namiki et al.; 2000), but its effects are restricted to CNS (Hiebert et al.; 2002). It has been demonstrated that BDNF is able to increase the number of contacts with propriospinal interneurons (Vavrek et al.; 2006). Indeed, it increases the growth of local motor, sensory, and coerulospinal axons. However, even though it is able to protect CST neurons (Hiebert et al.; 2002; Zhou et al.; 2003), it has no effect on their growth because its receptors are present only on the somata and dendrites but not on CST axons (Lu et al.; 2001). Overall, BDNF seems to be able to promote survival of axotomized axons, the regeneration of injured fibres and their re-myelination (a complete review could be found, Weishaupt et al.; 2012). Like NGF, neurons show responsiveness to BDNF also after a delayed administration (Koda et al.; 2002).

Neurotrophin 3 (NT-3) is a potent neurotrophic factor for ascending sensory axons (Taylor et al.; 2006; Bradbury et al.; 1999) and CST axons (Grill et al.; 1997; Schnell et al.; 1994). Adeno-associated vector secreting NT-3 (Fortun et al.; 2009) resulted in an increased number and sprouting of CST axons around the lesion, together with a decreased astrogliosis.

Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor- β (TGF- β) family. It is able to enhance axonal sprouting, synaptic connectivity and GABAergic

neurotransmission in SC after injury, resulting in an improved functional outcome (Koelsch et al.; 2010).

Vascular endothelial trophic factor (VEGF) has been reported to decrease CST degeneration and to decrease apoptosis (Facchiano et al.; 2002) after SCI. However, an increased secondary damage has also been reported after VEGF treatment, due to the increased angiogenesis and subsequent increased influx of inflammatory cells (Benton et al.; 2003).

Insulin growth factor (IGF-1), like BDNF, seems to be able to promote corticospinal neuron survival, but not their regeneration (Hollis et al.; 2009).

However, continuous trophic factor expression in the SCI site, even if necessary to stimulate the entry of axons into the graft, does not promote axonal growth toward the inhibitory environment beyond the lesion. This goal has been achieved by changing the temporal expression of growth factors, for example through the tetracycline-inducible expression of BDNF in modified fibroblasts (Blesch et al.; 2007). The authors demonstrated that transient growth factor delivery is sufficient to sustain long-term axonal projections. Whatever the method, the goal is to create a gradient of trophic factors, in order to guide axons, not only to regenerate into the graft, but also to grow out of it into the CNS environment.

7. SPINAL CORD INJURY – STATE OF THE ART

An important goal in the treatment of SCI is the development of effective treatments to prevent tissue damage and promote neuron survival. Indeed, up to now there are no efficient clinical treatments for SCI patients. The usual procedure provides stabilization and decompression of SC combined with high dose of methylprednisolone, the only approved treatment (Bracken and Holford et al.; 2002). However, there is no consensus about the true beneficial effects of these therapies so there is always the constant search for new treatments.

7.1 Surgical interventions

Following the lesion, the medical objective is to protect the traumatized region to reduce the possible compression, whose persistence is a source of neuronal tissue degeneration. This requires a well-timed treatment. In the medical-scientific context there is a general consensus that surgical decompression of the SC, compressed or bruised, is often necessary (Fehlings and Perrin.; 2005). This can be achieved mainly through the spinal traction and stabilization of the spine. The spinal traction is based on the manual or mechanical application of a force (usually a weight) along the longitudinal axis of the spine. The fracture of the spine vertebrae may be stabilized by the insertion of rods and screws to align correctly the spine. In addition, it may be necessary to

remove bone fragments or foreign objects in the SC with a surgical intervention. At a physiological level, surgical decompression has the ability to reduce the intradural pressure thus increasing the blood flow to the spinal cord, reducing ischemia and edema, and preventing secondary damage (Li et al., 2014).

Another surgical technique is intramedullary decompression: it has the potential to improve damaged vessels and prevent further cell death, but it is still under study (Li et al., 2014).

It is known that if the surgery is performed within 24 hours after the trauma, the chances of functional recovery increase significantly (Fehlings et al.; 2012). However, the optimal timing of surgical intervention generates considerable debate and remains unanswered. Although previously cases of post-operative complications were common, now they are much decreased thanks to centers specialized in the treatment of SCI (Cristante et al.; 2012).

Also in animal models, decompression of the spinal cord improves functional recovery and minimizes secondary injury after SCI (Carlson et al.; 1997, Dimar et al.; 1999).

7.2 Pharmacological interventions

Methylprednisolone

The only pharmacological treatment for the SCI acute phase so far accepted by the US Food and Drug Administration (FDA) is the systemic use of a cortisone based-drug, methylprednisolone (MP) (Bracken et al.; 1984, 1990, 1997). Its neuroprotective effects are due to its anti-oxidant activity, to the improvement of the blood flow of the spinal cord, to the reduction of calcium influx, to the decrease of the axonal retrograde degeneration and the reduction of lipid peroxidation. In a few years the North American Spinal Cord Injury Study (NASCIS) carried out 3 different studies of MP: the results showed that the neuroprotective activity, even though limited, is present only if high doses of MP are administered within 3-8 hours after the injury. However, high doses of MP were associated with pneumonia, infection, pulmonary embolism and death (Fehlings and Baptiste, 2005). Another two prospective clinical trials on the administration of MP in spinal injuries have been published (Pointillart et al.; 2000) in which some benefits were seen, but both of these studies involve a small number of patients and therefore the results are difficult to interpret (Hawryluk et al.; 2008). The data obtained led to a huge controversy about the use of MP in the acute phase of injury, which had a climax in 2002 with the conclusion of the American Association of Neurological Surgeons Congress on Neurological Surgery that the treatment with MP is

recommended for 24 or 48 hours in the treatment of patients with acute spinal cord injury.

A possible alternative approach could be the use of nanoparticles (NPs) as delivery tool for MP, in situ administered, to enhance the effectiveness of MP therapy and avoid the side-effects arising from high dose corticosteroid therapy. This treatment significantly decreases secondary injury-related inflammation, resulting in significantly reduced lesion volume and improved behavioral recovery (Kim et al.; 2009, Chvatal et al.; 2008, Cerqueira et al.; 2012).

Minocycline

Minocycline is a broad spectrum tetracycline antibiotic which shows also neuroprotective effects through the inhibition of M1 microglia/macrophages (Festoff et al.; 2006; Kobayashi et al.; 2013). Minocycline decreases also the formation of ROS, reduces matrix metalloproteinase activity and inhibits apoptotic cell death (Yong et al.; 2004). Minocycline is an attractive drug for clinicians because it is known to be well tolerated in human, where it has been used for acne treatment for decades.

A randomized Phase II trial has demonstrated that treatment with minocycline leads to improvement in functional outcome in patients with cervical motor-incomplete injury, although the improvement was not statistically significantly. The study also showed no serious adverse events in patients receiving treatment (Casha et al.; 2012). At present,

two Phase III clinical trials are in progress for patients with acute SCI (NCT01828203, NCT01813240 identified in ClinicalTrials.gov).

Additional pharmacological therapies that have been explored in human SCI trials, but have not demonstrated significant clinical efficacy include: naloxone (Bracken et al.; 1990), thyrotropin releasing hormone (Pitts et al.; 1995), trilazad mesylate (Bracken et al.; 1997), nimodipine (Pointillart et al.; 2000) GM-1 ganglioside (Geisler et al.; 2001), gacyclidine (Lepeintre et al.; 2004), and granulocyte colony stimulating factor (Yoon et al.; 2007).

Chronic treatments

Therapies in the chronic phase after a spinal cord traumatic event include drugs, rehabilitation, medical devices and functional electrical stimulation (FES). Drugs are used to control pain and spasticity, to improve bladder control and bowel and sexual functions. For example, gabapentin is used as an analgesic drug to reduce neuropathic pain in chronic patients with complete lesion.

Rehabilitation can be active, in which case it involves the SCI individual and volunteers making efforts to improve motor performance, or it can be passive, performed from massages and movements induced by the physiotherapist. Passive rehabilitation is a fundamental and essential part of any treatment protocol, but alone is not sufficient to maximize functional outcomes after injury. To date, however, no active

rehabilitation therapy has been completely validated as essential or effective for functional recovery following the various types of spinal lesion, although there is evidence that the effort may encourage the formation of new local connections in the CNS. Several computerized devices are used to help patients to become independent. Also functional electrical stimulation (FES) can be used to allow patients to control the muscles and organs.

7.3 Cell therapy

The cell transplantation therapy is one of the most promising strategies for the treatment of SCI and this is a focal point of some preclinical research. Recruited cell types are olfactory ensheathing cells (OECs), Schwann cells (SCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), oligodendroglial progenitor cells and embryonic stem (ES) cells. The beneficial effects of these therapies are multifactorial, because these cells are able to secrete several trophic factors with neuroprotective effect or promote neuronal plasticity.

Most transplants are performed directly in the wound site or in the areas adjacent to the lesion, through the injection of a few microliters of cell suspension. When the treatment is carried out in sub-acute or acute phase (transplantation takes place respectively immediately after the trauma or a few days after the injury) a poor outcome is obtained because the SCI site is a toxic and inhospitable environment for the transplanted cells.

The properties, advantages and disadvantages of each of these cells are further reviewed by Silva and collaborators (Silva et al.; 2014).

7.4 Molecular therapy

The remarkable advances in molecular biology in the past decades have led to a better understanding of the mechanisms implicated in the pathophysiology of SCI as well as mechanisms that control axonal growth and sprouting. This knowledge has raised the hope of developing new therapies that can attenuate secondary damage and maximize regeneration after a SCI. Molecular therapy usually focuses on the modulation of inflammatory response, administration of growth-stimulating factors and/or blocking the inhibitory nature of the injured adult CNS tissue.

Non-steroidal anti-inflammatory drugs (NSAIDs): The most studied among the NSAIDs for SCI is Indomethacin which produced a reduction of edema and tissue damage. Others NSAIDs tested are BW755C, NS-398, naproxen, aspirin, ibuprofen, administered a few minutes before or after the injury. Among them, ibuprofen appears to promote both histological and behavioral improvements following administration up to 3 and 7 days after injury (Wang et al.; 2009). Ibuprofen is able to reduce the RhoA activity by promoting axonal regeneration, as well as to alleviate the inflammation.

It has been long understood that neuronal damage is mediated by disproportionate intracellular accumulation of sodium and calcium ions. This accumulation is mediated by overstimulation of NMDA receptors (due to high glutamate levels after injury) in a process known as “excitotoxicity” (Agrawal and Fehlings, 1996; Faden et al.; 1989). In order to protect neural tissue several researchers explored the therapeutic potential of either sodium channel blockers, such as **riluzole**, or NMDA antagonists, such as **magnesium**.

Riluzole is a drug approved by the FDA for the treatment of amyotrophic lateral sclerosis (ALS) that can be administered orally. For years it has been known that damage to the white matter can be mediated by an influx of sodium ions through sodium channels and voltage-dependent block of these channels has been shown to significantly prevent the loss of white matter (Rosenberg and Wrathall, 2001). Riluzole appears to act by blocking voltage-dependent sodium channels, with a consequent decrease in the pre-synaptic release of glutamate. Its neuroprotective action is strongly linked to the time from injury to administration. It has proven effective if the dose is administered after 15 minutes or 30 minutes from the lesion (Stutzmann et al.; 1996). These studies report a significant reduction of secondary damage around the lesion and an improvement in the recovery of motor function. Having already been demonstrated as safe for humans and having achieved encouraging results in preclinical studies, riluzole is now being tested clinically. Currently, a prospective,

randomized, multicenter Phase II/III trial is ongoing in patients with acute C4-C8 SCI (NCT01597518 identified in ClinicalTrials.gov).

Magnesium has been investigated in animals as a neuroprotective agent for traumatic brain injury (TBI) and SCI (Suzer et al.; 1999; Kaptanoglu et al.; 2003; Gok et al.; 2007; Hoane et al.; 2007; Wiseman et al.; 2009). The biological rationale for magnesium in SCI includes the attenuation of excitotoxicity through a voltage-dependent blockade of NMDA receptors (Zhang et al.; 1996), and the replacement of depleted magnesium levels following injury (Vink et al.; 2000). In acute SCI animal models, magnesium sulfate administration has been shown to decrease membrane damage, protect axonal function and improve electrophysiology and behavioural outcome (Suzer et al.; 1999; Kaptanoglu et al.; 2003). However, the neuroprotective effect was achieved with extremely high doses of magnesium that far exceed the tolerable human dose. More recently, magnesium chloride formulated within polyethylene glycerol (PEG) has been developed, and the dose of magnesium is similar to the clinically-acceptable dose. This formula has been investigated as a potential neuroprotective agent for SCI in thoracic and cervical SCI animal models (Kwon et al.; 2009; Lee et al.; 2010). A clinical trial of a proprietary form of PEG with magnesium chloride, AC105 (NCT01750684 identified in ClinicalTrials.gov), is currently underway to determine the efficacy of the drug in patients who have a SCI.

7.5 Physical approaches

To minimize secondary damage to the spinal cord physical approaches may be possible among which the most studied are hypothermia, hyperbaric oxygen and training. The first technique involves local cooling by pouring a low-temperature saline solution. It is based on the fact that low temperature protects from phenomena of hypoxia and ischemia. However, being poorly reproducible and unreliable, it has a high mortality rate (Schwab and Bartholdi, 1996).

The second technique is characterized by raising the partial pressure of oxygen at the injury site, and this is achieved by making the patient breath inside a hyperbaric chamber with an oxygen pressure greater than that atmospheric (Yeo et al.; 1976). The rationale is that the loss of perfusion can be compensated by the partial pressure of oxygen (Cristante et al.; 2012) and in the literature there are numerous positive results obtained with this therapy (PEC Galvao and RP, 2011).

Finally, many studies show excellent improvements in coordination and in functional recovery by performing different types of exercise (Cristante et al.; 2012). Benefits have also been observed at the level of intrinsic neural circuits (AF Ferreira, 2012).

7.6 Combinatorial therapies

Because of the limited effective treatments for SCI, it is not surprising that many pharmacological and non-pharmacological approaches are

being investigated to provide better treatment for SCI patients. Although therapeutic effects have been observed in preclinical work, we have not seen any treatments translated successfully into clinical use. One of the reasons for this is the complexity and multi-factorial nature of the secondary pathophysiological changes following SCI. Most treatments only target one aspect of this response; however, a successful approach is likely to depend on a combination of different treatments. Single therapies have shown, so far, only limited effects, because it remains the need to overcome other toxic or inhibitory factors. In order to increase the small effect on behavior seen after a single neuroprotective treatment, the most powerful strategy is probably the simultaneous treatment of different pathological processes, as suggested by other studies (Thuret et al.; 2006). This should improve the effects of each therapy. However, in vivo combinatorial studies require a great effort, because introducing each additional treatment doubles the number of experimental groups and the total number of animals necessary to evaluate all the parameters singularly (Zhao et al.; 2013).

An exhaustive overview of studies using combinatorial strategies is in the review of Silva and collaborators (Silva et al.; 2014).

8. THE ROLE OF INFLAMMATION

In physiological conditions, the blood-brain barrier (BBB) limits the entry into the CNS of patrolling bone marrow (BM)–derived immune cells, while resident microglia perform physiological surveillance. In response to mechanical injury to spinal cord, microglia become rapidly activated, undergoing morphological and molecular changes often associated with neurotoxicity and initiating the inflammatory cascade that mainly characterizes the secondary injury. The composition and the potential effect of the cellular and molecular inflammatory cascade changes as a function of time and distance from the lesion.

The main pathological changes have been arbitrarily categorized into 3 stages by Shin et al.: (1) an early inflammatory stage, from the injury to the first 3 days, (2) a cleaning stage, approximately from 4 days to about 2 weeks post-injury and (3) reactive gliosis at 2 weeks post-injury (Shin et al.; 2013).

The first stage is characterized by hemorrhage, destruction of myelin, cell death, edema, and infiltration of inflammatory cells due to the BBB disruption. As residents of the SC, microglia and astrocytes are the first cells to respond to tissue damage. These cells are important for re-establishing tissue homeostasis (Davalos et al.; 2005, Myer et al.; 2006, Okada et al.; 2006). They are able to release chemokines and cytokines, both pro-inflammatory, such as tumor necrosis factor (TNF) α , interleukin (IL)-1 β , IL-6, interferon (IFN)- γ , and anti-

inflammatory interleukins such as IL-10 and tumor growth factor (TGF)- β 1, and other vasoactive substances, such as reactive oxygen species, kinins, histamines, nitric oxide and elastase, that enhance recruitment of peripheral leukocytes (Rice et al.; 2007, Weber et al.; 2007). The first circulating leukocytes that infiltrate injured tissue are neutrophils, followed by monocyte-derived macrophages (Stirling and Yong; 2008, Kigerl et al.; 2006). These cells support recovery processes through their ability to phagocytose cellular debris but they are also able to enhance the release of cytokines, free radicals, eicosanoids and proteases. Unlike neutrophils, macrophages persist in SCI lesions years and months respectively for humans and rodents (Kigerl et al.; 2006, Chang et al.; 2007).

Also T and B lymphocytes infiltrate the site of injury, but in fewer number and at later time than monocytes, and their functional significance remains a controversial issue (Ankeny and Popovich, 2009, Popovich and Longbrake, 2008). During the cleaning stage, edema is reduced and a massive proliferation of macrophages/activated microglia is identified in the site of lesion (Ahn et al.; 2012, Moon et al.; 2004). In the lesion core, macrophages remove cells and the myelin debris, reducing edema, and inducing the formation of a cavity (Blight et al.; 1985).

The third phase is characterized by astrogliosis and/or shrinkage of SC volume. Astrocytes become the predominant cell type (Fawcett et al.; 1999) and form a 'scar-like' barrier between the fluid-filled cavitation

and normal tissue (Popovich et al.; 1997). These aspects will be deeply discussed later in this thesis.

Recently, a later phase of cellular inflammation was observed persistent up to 180 days post injury (DPI), with a peak in the microglial/macrophage response at 60 DPI that is double the magnitude of the earlier peak. This later peak did not coincide with a change in locomotor function, suggesting that this second phase is insufficient to affect locomotor recovery (K. D. Beck et al.; 2010).

It has been demonstrated by previous studies that, despite variations in SCI lesions, the main features of neuropathological changes following SCI are similar among rodent models (Basso et al.; 1997) and human patients (Sala et al.; 1999, Metz et al.; 2000). However, species-specific differences in the distribution, magnitude, and composition of infiltrating leukocytes are present between humans and rodents, between rodent species (rat vs. mouse) and between different rat and mouse strains. The processes initiated by the pro-inflammatory cytokines, especially the activation of macrophages, exacerbate secondary tissue damage and promote the formation of an inhibitory glial scar (Rostworowski et al.; 1997, Popovich et al.; 2002). However, the role of neuroinflammation is still controversial, being both beneficial and detrimental for recovery after the trauma. Indeed, the immune response may facilitate the recovery from injury by reducing the size of the lesion, facilitating wound repair and stimulating axonal regeneration (Elkabes et al.; 1996, Bomstein et al.; 2003) (Streit et al.; 1998,

Klusman et al.; 1997). Moreover, further discussion concerning microglia/macrophages phenotypes, including classically activated M1 and alternatively activated M2 macrophages should be considered. Even though the M1 phenotype is usually associated with a neurotoxic effect, it is also involved in the axonal regeneration. In contrast, while the M2 phenotype is associated with neuroprotective effects, it lacks the necessary environmental signals to maintain the phenotype (Aguzzi et al.; 2013). It has also been reported that M1 phenotype shows an early and persistent activation after injury, while M2 protective phenotype has a transient expression and is significant only in a subacute phase of injury (David and Kroner, 2011, Kigerl et al.; 2009). The current view is that the inflammatory response after injury is a 'double-edged sword', having both neuroprotective and neurotoxic properties, and that the detrimental phases of inflammation are interspersed with neuroprotective events (Kwon et al.; 2004). From this perspective, a promising therapeutic strategy should limit the neurotoxic potential and enhance the reparative mechanisms.

To present, several attempts have been made to try and counteract the harmful effects of the inflammatory cascade, following different approaches, the main of which are: cellular, molecular, general immunosuppression and therapeutic vaccination.

Cellular approaches – These therapies target specific cell types which are involved in the spreading of injury. The selective depletion of neutrophils and/or monocytes leads to an improvement of functional

recovery and to a tissue sparing in rat models of SCI (Popovich et al.; 1999, Taoka et al.; 1997).

The blocking of leukocyte entry into the lesion site is achieved through the inhibition of leukocyte binding molecules, including selectins and integrins (Gris et al.; 2004, Mabon et al.; 2000, Bao et al.; 2004), or by the inhibition of chemokines or cytokines that enhance recruitment of leukocytes. MIP-II is a broad-spectrum chemokine receptor antagonist which is able to reduce leukocyte infiltration in rat models of SCI (Ghirnikar et al., 2000, 2001). Antibody to CXCL10 treatment decreases the number of macrophages, CD4+ and CD8+ T-cells and B-cells, with relevant effects on promoting axon sprouting and functional recovery in mice models (Gonzales et al.; 2007). Inhibition of pro-inflammatory cytokines, such as TNF- α with infliximab or genetic deletion, or IL-1 and IL-6 with receptor antagonist IL-1ra and IL-6ra, confer neuroprotection and ameliorate neuropathic pain, reduce apoptosis and inhibit p-38 MAP-kinase activation in rodents (Kim et al.; 2001, Vogel et al.; 2006, Okada et al.; 2004). In three different laboratories, selective cyclooxygenase (COX)-2 inhibitors, systemically administered, have demonstrated significant improvements in both behavioral and neuroprotective outcome measures (Hains et al.; 2001, Resnick et al.; 1998) in SCI rodent models.

On the other hand, Schwartz's laboratory has published numerous studies documenting the beneficial effects of enhancing inflammation, in

a carefully controlled way, after CNS injury. They demonstrated that, when pre-activated macrophages were implanted into the transected rat spinal cord, there was enhanced axonal regeneration and functional recovery (Rapalino et al.; 1998) even though the degree of regeneration was not better than using transplants of other cell types. Schwartz's group also showed that the macrophages were fundamental in the clearance of debris and this allowed axonal regrowth in transected rat optic nerves (Lazarov-Spiegler et al.; 1996, 1998).

Other researchers have called for caution in applying activated macrophage transplantation in a clinical setting. Moreover, Popovich's group identified different populations of macrophages - bone marrow-derived, resident peritoneal, and thioglycollate-elicited - that differentially contribute to secondary damage (Popovich et al.; 1999, Longbrake et al.; 2007).

In addition, it has been observed that the timing of intervention can significantly affect microglia/macrophage activation and change the functional outcome in mice models of SCI (Klusman et al.; 1997), raising the questions of which cell type is neuroprotective and when the best time of intervention is.

Molecular approaches - This kind of approach targets intracellular signaling pathways. After SCI, activated (phosphorylated) p38 MAP-kinase is rapidly increased. Some preclinical studies showed that a single dose of SB203580 inhibits phosphorylation of p38 MAP-kinase,

and reduces apoptosis and iNOS gene expression (Wang et al.; 2005, Xu et al.; 2006). More controversial are the effects after continuous intrathecal delivery (Horiuchi et al.; 2003, Stirling and Yong, 2008).

Initially, the Rho GTPase and its downstream effector, Rho kinase (ROCK) have become attractive targets because they were able to stimulate axonal regeneration, as will be discussed subsequently in this thesis. Surprisingly, inhibition of ROCK using Y27632 (Chan et al.; 2005) or fasudil (Nishio et al.; 2006, Sung et al.; 2003) came out to have also anti-inflammatory effects in models of SCI. Indeed, inhibition of this pathway may contribute to neuroprotection via suppression of immune cell migration and infiltration into the injury site. In vitro, the Y27632 decreased the number of macrophages that migrate in a three dimensional assay (Nakayama et al.; 2005) and impaired lymphocyte (T cell) proliferation (Tharaux et al.; 2003). In vivo, ROCK inhibition decreased leukocyte infiltration into the injured SC (Hara et al.; 2000) and decreased the cytokine production (Angkachatchai et al.; 1999). However, negative effects associated with ROCK inhibition have also been reported, because certain dosages of Y27632 actually decreased axonal sprouting/regeneration and impaired functional recovery (Chan et al.; 2005, Sung et al.; 2003).

Another attempted approach has been the selective inactivation of astroglial transcription factor nuclear factor (NF)- κ B, which is highly activated after trauma, and leads to both protective and detrimental effects. Brambilla et al. demonstrated in their work that transgenic mice

expressing an inhibitor of NF- κ B α , under the control of an astrocyte-specific promoter, leads to a dramatic improvement of functional behavior, suggesting that selective inhibition of NF- κ B signaling in astrocytes results in protective effects after SCI (Brambilla et al.; 2005).

General immunosuppression - Immunosuppressive approaches have been carried out by using inhibitors of calcineurin, such as cyclosporine A (CsA) and FK506 (Tacrolimus), that have proved to reduce neuroinflammation and to improve recovery (Guzmán-Lenis et al.; 2008, Kaymaz et al.; 2005, Hayashi et al.; 2005).

Intravenous application of an antiangiogenic compound CM101, one hour after SCI in mice, with the intent of preventing inflammatory angiogenesis and inhibiting gliosis, demonstrated an improvement of locomotion and appeared to be neuroprotective (Wamil et al.; 1998).

Other immunosuppressants, like minocycline or methotrexate, have been showed to be neuroprotective and to ameliorate neuropathic pain, basically suppressing macrophages/microglia activation, in addition to their antiapoptotic properties (Festoff et al.; 2006, Scholz et al.; 2008). The promising preclinical results about minocycline treatments have led to two clinical trials, one is currently recruiting participants (ClinicalTrials.gov Identifier: NCT01828203, NCT01813240).

Therapeutic vaccination - In the perspective of the dual role of inflammation, to find an effective way to boost the spontaneous neuroprotection, the effectiveness of therapeutic vaccination has been tested. Schwartz's group reported beneficial results. They showed that injection of anti-myelin basic protein (MBP) T cells (T-MBP) was neuroprotective in a model of SCI. Since this approach could provoke an autoimmune reaction, they tested a synthetic four amino acid peptide, copolymer-1 (Cop-1), which mimicked the MBP molecule but was non-encephalitogenic. This peptide was able to induce a T cell response against MBP, reporting neuroprotective effects (Hauben et al.; 2000). A patent for the use of Cop-1 and related peptides and T cells treated with these peptides was obtained by this group in 2005 and the treatment is expected to enter Phase II clinical trial, even if this approach is still controversial (reviewed in Chan et al.; 2008). Other groups reported that implantation of activated macrophages or T-lymphocytes activated against modified myelin self-antigens, achieved by either adoptive transfer or active immunization in rats (Hauben et al.; 2000, 2001, Moalem et al.; 2000), enhances recovery in SCI by conferring neuroprotection or regeneration. Although the intriguing concept of neuroprotective autoimmunity is growing in popularity, to date there is no definitive mechanism that explains its efficacy.

9. DRUG DELIVERY TO THE SPINAL CORD

Common practice methods to administer pharmacological compound in CNS are based on oral, intravenous (IV) and intraarterial (IA) delivery. However, many limitations drastically reduce the applicability of the above mentioned route of administrations: i) limited access to the SC environment for the physical obstruction due to the blood spinal cord barrier (BSCB), ii) the half-life of the drug in the plasma and iii) potential unacceptable side effects of prolonged treatments. Consequently, new efforts are directed to develop innovative strategies and drug delivery tools to overcome BSCB or perform effective drug delivery in either cerebrospinal fluid (CSF) or SC parenchyma.

9.1 Intrathecal drug delivery: catheters and minipumps

Intrathecal (IT) drug delivery by catheters and implanted minipumps has been clinically used as an alternative route of drug administration to the systemic and oral ways, expanding the medical options to treat the SC (Simpson et al.; 2003). IT delivery is proposed as way to release compounds with a single or continuous infusion directly into the CSF. Indeed, a direct injection of drug is clinically possible into the spinal space corresponding to the interstice between the pia mater and the arachnoid membrane, without great iatrogenic exacerbation of the patient's medical condition (Lawson et al.; 2012). IT delivery shows

various additional therapeutic advantages compared to conventional routes: a more localized immediate pharmacological activity, a greater control of drug delivery, rapid reversibility and reduced side effects (Smith et al.; 2008). However, a direct injection or infusion in the CSF shows a poor distribution of therapeutic compounds into the SC parenchyma, reaching only the outer part of it. Furthermore, CSF is produced in the choroid plexus in the brain and moves in a pulsatile manner to the SC; CSF then returns to the vascular system by venous sinuses via the arachnoid granulations (Pardridge et al.; 1997). CSF is produced at a rate of 0.2 - 0.7 mL/min and it is renewed within 5h. For these reasons the injected therapeutic compounds return quickly in the bloodstream and the BSCB again represents a strong limitation to a diffuse pharmacological treatment of the SC.

In addition, some side effects regarding the placement of the catheters were described, such as obstruction, leakage, breakage, dislodgment, side effects of the surgery required for the placement and pump refilling. Hemorrhage, CSF leaks and infections, such as granulomas, were observed too (Belverud et al.; 2008).

9.2 Nanoparticles

In recent years, nanomedicine has provided many innovations and has been increasingly applied in drug delivery, underlining the importance of NPs in biomedical applications.

The science involved in their synthesis is nanotechnology, defined as “the engineering and manufacturing of materials at the atomic and molecular scale”. In its strictest definition from the National Nanotechnology Initiative, nanotechnology refers to structures roughly in the 1–100 nm size regime in at least one dimension. Despite this size restriction, nanotechnology commonly refers to structures that are up to several hundred nanometers in size. Recently, significant achievements have been obtained in developing NPs as new smart tools able to deliver in situ a wide range of molecules (drugs, antibodies and neurotrophins). Indeed, due to their versatility in terms of size, potential surface and hydrophilic or lipophilic characteristics, polymeric NPs lead relevant advantages in drug delivery by increasing the selectivity of drugs and by controlling their release overtime (Cho et al.; 2008, Kohane et al.; 2007, Mout et al.; 2012). Furthermore, to obtain NPs able to pass biological barriers, various materials and synthetic approaches are being investigated. Several cell-penetrating peptides, such as transactivating – transduction peptide, were found to be capable of penetrating the barrier, and their attachments to the surface of liposomes and NPs were used to facilitate internalization of these nanostructures into the CNS (Begley et al.; 2004, Liu et al.; 2010). Moreover, poly(butyl cyanoacrylate) NPs can penetrate the barrier via apolipoprotein-mediated transport, showing neuroprotective efficacy once functionalized with enzymes (superoxide dismutase) and antibodies (N-methyl D-aspartate receptor 1) (Reukov.; 2011).

Furthermore, NPs are considered a primary vehicle for targeted therapies because of their ability to enter and distribute within cells by energy-dependent pathways. So far, many studies have shown that NP properties, such as size and surface, can influence how cells internalize and take them up. Once taken up, NPs may act as a drug depot within cells and could be useful in achieving therapeutic dosing via targeted therapies. Furthermore, NPs can sustain drug release profile overtime and protect therapeutic compounds from efflux or degradation (Cerqueira et al.; 2012). Receptor mediated endocytosis could be the potential filter for greater selectivity in cellular targeting. The cellular membrane is dotted with a myriad of receptors, which extracellularly interact with their respective ligands (or with NPs whose surface is functionalized with ligands) transducing a signal to the intracellular space. This signal can trigger a multitude of biochemical pathways and, furthermore, it may also cause internalization of the ligand and its appended NPs via endocytosis.

However, NPs sometimes limit their therapeutic efficacy because of no specific uptake by macrophages (Lunov et al.; 2011).

One attractive alternative, to reduce the uptake kinetics, is to functionalize NPs with synthetic polymers creating covalent bonds: polyethylene glycol (PEG) is the most widely used polymer for this purpose. The attachment of PEG chains to NPs can sterically hinder its access to macrophages surface receptors and subsequently delay their uptake: PEGylated NPs have an increased half-life in the bloodstream.

Further, in vivo paradigms showed that PEGylated NPs also significantly reduced the formation of reactive oxygen species and the process of lipid peroxidation of the cell membrane. Moreover, the use of NPs can also optimize the delivery of MP, the only FDA approved drug for SCI treatment, providing a diffusive barrier and enhancing its neuroprotective properties (Kim et al.; 2009). The use of NPs as carriers for neuroprotective agents was also considered for prostaglandin E1: its sustained delivery significantly prevented cell death, induced angiogenesis and improved blood flow, thereby preserving the remaining cells and recruiting them in spinal diseases. In addition, hepatocyte growth factor released through NPs contributes to neuroprotection, anti-apoptosis and angiogenesis around a SCI lesion site. Furthermore, NPs can be used to deliver drugs selectively into specific cells of the spinal cord, paving the base for a cell-targeted therapy. In addition, nanostructured materials could be the right answer to the multitherapeutic clinical needs. Indeed, a therapeutic combinatorial treatment is a new challenge in the pharmacological management of SCI and simultaneous drug delivery through nanovectors could represent a considerable opportunity to synergy the efficacy of therapeutics against the multifactorial mechanisms of the secondary injury.

9.2.1 Nanoparticles and SCI

Different preclinical studies have demonstrated that NPs represent a promising drug delivery alternative for SCI treatment. Chemically conjugated NPs composed of ferulic acid and glycol chitosan, systemically administered in SCI rat model, were able to reach the lesioned spinal cord showing neuroprotection and functional restoration (Wu et al.; 2014). Furthermore, intravenously injected NPs containing prostaglandin E(1) were able to reduce the lesion cavity volume and promote the recovery of motor dysfunction (Takenaga et al.; 2010). A preclinical study using GDNF loaded in poly-lactic-co-glycolic acid (PLGA) NPs, directly injected into the damaged spinal cord to target neural and glial cells, demonstrated an increase of neuronal survival and an improvement of motor locomotion (Wang et al.; 2008). In addition, nanovectors were used to optimize the delivery of anti-inflammatory drugs. This is the case of MP loaded in PLGA and administered in situ (Kim et al.; 2009).

It showed a higher pharmacological efficacy compared with the conventional systemic administration, reducing tissue damaging and inflammation, and improving behavioral outcome in an SCI rat model (Kim et al.; 2009). Furthermore, a micellar structure of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) has been used as a delivery vehicle able to increase the bioavailability of MP in the injured SC (Chen et al.; 2008). Also the delivery of cerybrolysin (a mixture of different neurotrophic factors such as BDNF, GDNF, NGF, and ciliary

neurotrophic factor) was able to reduce spinal cord water content, leakage of plasma proteins, and neuroprotection when carried by NPs (Menon et al.; 2012). The direct injection of NP suspension into the injury site is one attractive alternative compared with systemic administration. Indeed, intravenous administration of NPs could have different contraindications for the treatment of the SCI, such as limited or unequal distribution of NPs in the target tissues (owing to BSCB restriction) (Rossi et al.; 2013), and relevant NPs uptake by circulating macrophages in the liver and/or spleen. Furthermore, NPs intrathecally injected without any support very often leave the zone of injection. According to these critical issues, several studies suggested to associate hydrogels with NPs, providing a localized targeted therapy able to maximize the efficacy of neuroprotective agents (Kang et al.; 2013, Baumann et al.; 2010).

Kang et al showed that NGF-2 encapsulated in PLGA NPs and embedded in a biopolymer blend of hyaluronan and methylcellulose was able to enhance the endogenous angiogenic response once implanted into the damaged spinal cord (Kang et al.; 2013). Moreover, *in situ* delivery was optimized by encapsulating MP in PLGA-based NPs subsequently embedded in an agarose hydrogel and implanted into the site of the contusion. This showed a significant reduction of the early inflammation (Chvatal et al.; 2008). Interestingly, nanomaterials themselves have been recently recognized as having a neuroprotective efficacy. Intravenously injected micelles composed of self-assembled

monomethoxy poly(ethylene glycol)–poly(d,l-lactic acid) diblock copolymer effectively recovered locomotor function and reduced both the lesion volume and the inflammatory reaction in SCI rats (Shi et al.; 2010). Moreover, local administration of poly(ethylene glycol) reduced oxidative stress and repaired nerve membranes, leading to the restoration of the nerve potential conduction and increasing behavioral outcome in SCI models in guinea pigs (Borgens et al.; 2002). A superior neuroprotective efficacy has also been demonstrated using nanowired material as a delivery tool in SCI. Tian et al. showed that TiO₂ nanowires were able to increase the bioavailability of neuroprotective and anti-inflammatory compounds (Acure Pharma synthesized compounds), improving their efficacy. This was likely due to the higher concentration of drugs available in the injured tissue (Tian et al.; 2012). NPs thus appear to be a very interesting delivery tool also for a cell-targeted therapy given their ability to enter in specific cells, exploiting specific receptors or permissive pathways. Once internalized, NPs may act as a drug depot within cells, protecting therapeutic compounds from degradation or efflux and delivering therapeutic doses with a sustained-release drug profile. An interesting application of this innovative cell-targeted delivery regards the pharmacological modulation of microglia/macrophages. New evidence both *in vitro* and *in vivo* suggests that NPs can be selectively internalized by a specific endocytotic/phagocytic activity of macrophages after different insults, exploiting them as Trojan horses (Jain et al.; 2013). It is well known

that microglia/macrophages assume phagocytic activity after traumatic stimuli and this makes NPs a potential tool for drug targeting. Recently, this approach has been used by Cerqueira et al. They demonstrated that surface-engineered carboxymethyl chitosan/ polyamidoamine dendrimer NPs were able to deliver MP into glial cells, specifically microglial cells, allowing a controlled and selective release of MP in the injury site. However, a critical issue in using NPs is the safety of the nanostructured material proposed as biomedical tools. Indeed, biocompatibility and efficacy can be influenced by minor variation in different parameters that characterize the nanomaterial, such as size, shape, chemistry, solubility and surface area (El-Ansary et al.; 2013), suggesting that a deeper investigation is mandatory in these characteristics before being translated into clinical trials and medical practice.

10.OVERALL OBJECTIVES AND SPECIFIC AIMS

Spinal cord injury is characterized by a primary event, due to a mechanical insult to the spinal cord, followed by the so called "*secondary injury*", that exacerbates the clinical outcome. *Secondary injury* includes a wide spectrum of neuropathological events, resulting in motor dysfunction below the level of lesion. Much evidence shows that persistent inflammation, represented by immune cell infiltration and release of inflammatory mediators, is a crucial event involved in worsening of the *secondary injury*. In particular, microglia/macrophage related inflammation appears to be a self-propelling mechanism which leads to progressive neurodegeneration and development of persisting pain state in SCI.

So far, the challenge has been to find ways to selectively modulate the microglia/macrophages activation after SCI, in order to understand the real involvement of these cells in the inflammatory response occurring during and after the trauma. Indeed, the role (beneficial or detrimental) of the inflammatory response associated with the activation of microglia/macrophages during SCI repair remains unclear. The magnitude of SCI pathology and related functional loss correlates with a robust microglia/macrophages response and the blockade of their own related inflammatory signaling often confers neuroprotection (Festoff et al.; 2006, Donnelly et al.; 2011). Contrarily, different studies showed that activated microglia/macrophages in particular conditions could

ameliorate the progression of SCI (Shechter et al.; 2013, Shechter et al.; 2009). Different reasons could explain the difficult interpretation of these inflammatory events: 1) high difficulty to target experimentally microglia/macrophages; 2) beneficial or detrimental effects are spatially and temporally defined; 3) different subsets of microglia/macrophages are involved (M1 and M2 phenotype).

Growing interest in the improvement of in situ drug delivery system to treat SCI was demonstrated in the last years (Perale et al.; 2011) and the development of new tools for enhancing drug delivery in specific target cells is one of the most promising challenges. Recent advances in polymer science have provided a huge amount of innovations, underlining the increasing importance of NPs in this field (Perale et al.; 2011, Mout et al.; 2012). NPs are considered a primary vehicle for targeted therapies because they can pass biological barriers and enter and distribute within cells. New evidence *in vitro* and *in vivo* suggests that NPs can be selectively internalized by a specific phagocytic activity of macrophages after insults, exploiting them as Trojan horses to selectively treat them. Many anti-inflammatory treatments have been proposed to modulate microglia/macrophages in SCI, but no one is able to do that in a selective way (Festoff et al.; 2006, Loane et al.; 2010, Wells et al.; 2003, Gwak 2012) and this may give rise to potential effects associated with other cells of the central nervous system, leading to misinterpretation.

The innovative selective delivery of minocycline using polymeric NPs as carriers, could represent a new promising strategy to gain new insights into cellular inflammation of SCI.

In order to increase the efficacy of potential drugs (e.g. minocycline) in modulating macrophage/microglia mediated inflammation and deeply understand the role of these cells in the inflammatory process after SCI, the aim of this project is to develop a new drug delivery system for a selective pharmacological treatment of activated macrophage/microglia and to evaluate its efficacy.

SPECIFIC AIMS

The characterization of this new drug delivery tool will be achieved through the development of the following points:

- The first part of the work aims to characterize a new nanoparticle tool able to treat selectively activated microglia/macrophages. Different polymeric nanoparticles will be tested *in vitro* and *in vivo* to define the most suitable biocompatible and biodegradable NPs, able to release loaded drugs within cells, after their uptake.
- In the second part of the work, polymeric NPs characterized previously will be loaded with a well-known anti-inflammatory drug, minocycline. Minocycline-loaded NPs will be used *in vitro*

and *in vivo* to evaluate the ability of this treatment in modulating microglia/macrophages activation.

- The third part of this work aims at understanding whether the specific inflammatory response related to microglia/macrophage activation could lead to an amelioration of the behavioral outcome of SCI in a mouse model at different times of investigation (subacute and acute phase) by means of a selective modulation of this cellular response. In addition, the molecular mechanisms related to the inflammatory activation of microglia/macrophages will be investigated after their selective modulation.

G. MATERIALS and
METHODS

1. BIOMATERIALS SYNTHESIS

1.1 NPs SYNTHESIS (Provided by Politecnico di Milano)

The macromonomer used for the NPs synthesis was synthesized through a ring opening polymerization reaction (ROP) using a procedure reported in literature (Ferrari et al.; 2013). PCL based NPs were synthesized through free radical emulsion polymerization, by using KPS as initiators and Tween80 as surfactant. RhB was used as fluorescent dye to detect NPs, it was covalently bonded to HEMA (2-hydroxyethyl methacrylate), an FDA approved molecule, through an esterification reaction and then copolymerized with other monomers obtaining NPs with RhB covalently linked to the polymer matrix.

1.2 LOADING OF MINOCYCLINE IN PCL NPS (provided

by Politecnico di Milano) Since NP synthesis has been carried out using an emulsion polymerization process, the drug loading step could not take place during NP formation because of the presence of both high temperature and radicals; therefore, a postsynthesis process has been set up. First NPs latex concentration was increased up to 5% w/w under a vacuum; the absence of NPs coalescence during this step was confirmed by Dynamic Light Scattering measurements.

Minocycline-base was preliminarily obtained from minocycline hydrochloride (Sigma-Aldrich, CAS 13614-98-7) in order to increase its hydrophobicity and consequently the loading efficiency. Minocycline hydrochloride was converted into its free base before being loaded within NPs to increase its hydrophobicity and consequently the percentage of loading. Briefly, minocycline hydrochloride (10 mg) was dissolved in a saturated solution of sodium bicarbonate (2 mL). Then the aqueous solution was extracted four times with dichloromethane (4 ± 2 mL). The organic phase was separated and then dried over sodium sulfate, filtered, and concentrated under reduced pressure. The resulting powder (8 mg, 80% yield) was then dissolved in Dimethyl sulfoxide at a concentration of 2 mg/mL. The drug loading procedure is facilitated since the NPs are naturally constituted of a hydrophobic core and a hydrophilic shell constituted of PEG chains. The drug was entrapped in the partially swollen preformed NPs in the following way: a Polytetrafluoroethylene cylinder of 1 cm of diameter and 1 cm of length with an axial perforation of 1 mm diameter and a radial one of 500 μm was used as a mixing device for the drug loading procedure. The NP latex (300 mg in 6 mL of deionized water,

concentration of 50 mgNPs/mLwater) and the dissolved drug (2 mg of minocycline in 1 mL of DMSO) were loaded in syringe pumps and injected radially in the device at a flow rate of 30 and 5 mL/min, respectively. Four-hour dialysis was employed to remove free minocycline. The amount of minocycline present in PLC based NPs at the end of the dialysis period was determined by HPLC-MS/MS. For that, aliquots were added with IS and 10 volumes of chloroform:isopropanol (1:1), shaken for 5 min and centrifuged at 13.000 rpm for 10 min. Supernatants were dried under a gentle nitrogen stream at room temperature, and samples were finally reconstituted in acetonitrile (ACN, Sigma-Aldrich) containing 0.1% formic acid to be injected in the HPLC_MS/MS system maintained at 6°C. Stock solutions of minocycline hydrochloride (Sigma-Aldrich) and demeclocycline hydrochloride (Sigma-Aldrich), used as internal standard (IS), were prepared in methanol and kept at -20°C until use. All chemicals (formic acid, isopropanol, and chloroform, Carlo Erba) used were of analytical or HPLC-MS grade. The HPLC system consisted of an Alliance separation module 2695 coupled with a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters) controlled by Mass Lynx software

4.1. The mass spectrometer operated in positive ion and multiple reaction monitoring (MRM) mode, measuring the fragmentation products of molecular ions of substances. The instrument is equipped with an electrospray ionization interface and uses argon as collision gas. Source and desolvation temperatures were set at 100 and 300°C, respectively. Samples were analyzed with the ion spray needle operating at 4.8 kV, the cone voltage at 25 V, and the collision energy at 15 eV. The principal ion transition 458.1 > 441.2 was selected for minocycline quantification, while the transition 465.0 > 448.3 was selected for IS. Chromatographic separation was achieved on a Waters XTerra MS C18 column (100±2.1 mm, 3.5 µm) coupled with an XTerra C18 cartridge, held at 30°C. The mobile phases consisted of water (MP-A) and ACN (MP-B), both containing 0.1% formic acid. The HPLC system was set up to operate at a flow rate of 0.2 mL/min at a the following linear gradient: from 2 to 98% MP-B in 15 min, at 98% MP-B for 3 min, from 98 to 2% MP-B in 1 min (total run time 30 min). The retention times were 9.9 min for minocycline and 11.3 min for IS.

1.3 HYDROGEL SYNTHESIS (provided by Politecnico di Milano) Hydrogels (AC) were prepared by blocking

polymerization in PBS at about 80°C, where polymeric solution (carbomer 974P 0.5 wt.%, agarose 0.5 wt.%) was achieved by mixing polymer powders into the selected solvent, adding a mixture of cross-linking agents primarily made of propylene glycol (30 wt.%) and glycerol (1.2 wt.%). Reaction pH was indeed kept neutral. Gelation start was achieved by means of microwave (EM) stimulation. Effective gelation and reticulation were achieved by microwave heating for 1 min per 10 mL of polymeric solution.

2 IN VITRO EXPERIMENTS

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that comply with national (D.L. n. 116, G.U., suppl. 40, Feb. 18, 1992, Circolare No. 8, G.U., July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

2.1 PRIMARY CELL CULTURES Primary cultures of microglia or neuron/glia co-cultures were obtained from the spinal cord of C57BL/6J (Charles River Laboratories International, Inc.) and B6.129P-Cx3cr1tm1Litt/J (The Jackson Laboratory) mouse embryos (E13). Spinal cords were dissected, exposed to DNase and trypsin (Sigma-

Aldrich, Milan, Italy), and centrifuged through a BSA cushion. Cells obtained at this step were a mixed neuron/glia population and underwent centrifugation through a 6% iodixanol (OptiPrep, Sigma-Aldrich) cushion to separate large neurons from glial cells. The glial feeder layer was prepared by plating the glial fraction at a density of 25 000 cells/cm² into 12-well plates or into flasks, both previously pre-coated with poly-L-lysine (Sigma-Aldrich). Purified microglia were obtained from flasks containing confluent mixed glial cultures after overnight shaking at 275 rpm in incubators. The supernatants (containing microglia) were collected and seeded at a density of 20.000 cells/cm². Astrocyte cultures were obtained by treating the glial cultures, from which microglia had been previously harvested, with 60 mM L-leucine methyl ester (Sigma-Aldrich) for 90 min. To establish neuron/glia co-cultures, the neuron-enriched fraction (obtained from the iodixanol-based separation) was seeded at a density of 10.000 cells/cm² onto a mature astrocyte layer, and microglia were added (10% of the astrocyte number) on the third day *in vitro*.

2.2 CULTURE TREATMENTS Microglia activation was induced by exposing purified microglia cultures or neuron/glia co-cultures to 1 $\mu\text{g/mL}$ of LPS (from *Escherichia coli* 0111:B4; Sigma-Aldrich) for 18 h. Cultures maintained with normal medium served as the control condition. PCL NPs were then added to untreated or LPS-stimulated cultures. For the chlorpromazine hydrochloride (CPZ; Sigma-Aldrich) treatments, different solutions of CPZ (final concentrations: 30, 40, or 50 μM) were added to the culture medium 2 h before NPs exposure. CPZ did not show neurotoxicity up to 40 μM .

2.3 CELL VIABILITY ASSAY The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTT; Promega, Italy) was used according to the manufacturer's instruction to determine cell viability in NPs treated microglia cultures.

2.4 IMMUNOCYTOCHEMISTRY Cells were fixed with 4% paraformaldehyde and permeabilized by 0.2% Triton X-100 (Sigma-Aldrich). Staining was carried out by overnight incubation with the primary antibodies, followed by incubation with an appropriate fluorescent secondary antibody. Cell nuclei were labeled with

Hoechst 33258 (Invitrogen) by incubation with a 250 ng/mL solution. The following primary antibodies were used: anti-NeuroFilament 200 (rabbit, 1:500; Sigma-Aldrich); anti-GFAP (rabbit, 1:500; Millipore); anti-CD11b (rat, 1:1,000; eBioscience Inc.). Appropriate fluorescent secondary antibodies (Dy-light; Rockland Immunochemicals Inc.) conjugated to different fluorochromes were used at 1:1.000 dilution. For lysosomal activity analyses, a lysosensor dye (1:20.000 dilution, Life technologies) was used. For the membrane staining, we used the Wheat germ agglutinin (WGA) Alexa fluor 350 conjugated (5 μ g/ml, Life technologies).

2.5 TRANSMISSION ELECTRON MICROSCOPY A 5 μ L drop of purified PCL in PBS were placed on a 100 mesh formvar/carbon coated copper grid (EMS, Hatfield, PA, USA) and observed with an Energy Filter Transmission Electron Microscope (EFTEM, Zeiss Libra® 120) equipped with YAG scintillator slow scan CCD camera. Microglia cells were plated on glass coverslips and exposed to PMMA for 1 and 6 days. After incubation, cells were pre-fixed with 4% paraformaldehyde and 1% glutaraldehyde in Hepes 0,2M (pH 7.4) and fixed at 4°C in 1% glutaraldehyde in Hepes 0.2M, pH 7.4 until use. After 30

minutes incubation with osmium 1% in 0.12M phosphate buffer pH 7.4 on ice, cells were incubated with a saturated solution of thiocarbohydrazide followed by 1.5% ferrocyanide and 1% osmium for 30 minutes. Microglial cells were then counterstained with 0.5 % uranyl acetate after dehydration in graded series of ethanol and embedded in Epoxy medium (Epon 812 Fluka, Sigma Aldrich). Thereafter, coverslip was removed from the well of multiwell plate and, to allow the transfer of cells from coverslip to resin block, was placed on disposable flat embedding mold (Electron Microscopy Sciences) pre-filled with Epoxy resin and polymerized at 60°C for 72 h. After removing the glass coverslip using 40% fluorhydric acid, resin block with neurons on the top was trimmed to obtain a small pyramid suitable for ultrathin (55-60 nm thick) sectioning with a Leica EM UC6 ultramicrotome. Sections were then collected on 100 mesh formvar carbon coated grids and examined with an EFTEM equipped with a YAG scintillator slow scan CCD camera (Zeiss).

2.6 CHEMOTAXIS ASSAY Conditioned media (CM) were collected from purified microglia cultures treated with 1 mg/mL LPS (from *Escherichia coli* 0111:B4; SigmaAldrich) for 18 h to induce microglia activation,

and then exposed to empty NPs (PCL, NPs 0.25 gr polymer/mL) or minocycline loaded NPs (PCL-mino, 0.25 mg of minocycline within 0.25 gr of polymer/mL) for 72 h. CM were analyzed to detect the levels of CCL2 release by solid-phase sandwich enzyme-linked immunosorbent assay (ELISA, sensitivity of the kit: 15 pg/mL; eBioscience) following the manufacturer's instructions and stored at -8°C for macrophage treatments. Macrophages plated into a small slit of the m-Slide Chemotaxis 2D (IBIDI) were exposed to CM on the first day in vitro for 18 h. CM (final dilution 1:2.5) was added into the reservoir specifically used to test the chemoattractant properties of compounds, as described by Zengel et al. [34]. Each m-slide contains three independent experimental set up: control condition, where both reservoirs were filled with unconditioned medium (UM) and two testing conditions, where one reservoir was filled with CM (from LPS-activated microglia treated with empty PCL or PCL-mino) and the other one with UM. The evaluation of macrophages migration was performed using live cell imaging techniques. Time-lapse video microscopy was performed using a Cell[^]R imaging station (Olympus) coupled to an inverted microscope (Olympus IX 81) and equipped with

an incubator to keep constant temperature (37 °C) and CO₂ (5%). Images were acquired with a 4X objective 1 frame/30 min for a total of 18 h. Quantification of chemotaxis was performed by evaluating M1 macrophages migration, expressed by COM (Chemotaxis and Migration Tool, IBIDI) a strong parameter for evaluating chemotaxis that represents the spatial averaged migration of all cell endpoints.

3 IN VIVO EXPERIMENTS

3.1 SURGERY C57BL/6J mice (Charles River Laboratories International, Inc.) or B6.129P-Cx3cr1tm1Litt/J mice (The Jackson Laboratory) were used for in vivo studies. Before surgery, the animals received an antibiotic and analgesic treatment, respectively with a subcutaneous injection of ampicillin (50 mg/kg) and buprenorphine (0.15 mg/Kg). Entire surgical procedure was carried out in deep anesthesia by ketamine hydrochloride (IMALGENE, 100mg/kg) and medetomidine hydrochloride (DOMITOR, 1mg/kg) intraperitoneally. Animals were placed on a Cunningham Spinal Cord Adaptor (Stoelting, Dublin, Ireland) mounted on a stereotaxic frame, and laminectomy of T12 vertebra was done to uncover the

lumbar spinal cord. For the induction of a localized inflammatory reaction, using a glass capillary ($40 \pm 2 \mu\text{m}$ diameter) the hydrogel AC solution ($0.5 \mu\text{L}/\text{site}$) was injected in the spinal cord with a flow rate of $0.2 \mu\text{L}/\text{min}$. Stereotaxic coordinates were referred to the midline of the dorsal horn of the spinal cord. The needle was positioned at $+0.5 \text{ mm}$ aside from the midline, then it was deepened into the parenchyma to 0.7 mm below the pia mater. The injector was left in place for 1 min and then retracted for 0.2 mm before starting the delivery. After gel injection, the needle was left in place for an additional 3 min and then gently withdrawn. Mechanical trauma of the Spinal Cord at T12 vertebral level was achieved using an aneurysm clip with a closing force of 60g (left in place for 1 min and then removed). After spinal cord compression, dorsal muscles were juxtaposed using absorbable sutures and the skin sutured. Manual compression of the bladder was also performed twice daily for two days after SCI induction. At several time points mice were treated with intraparenchymal injections of minocycline loaded NPs (PCL-mino, 0.25 mg of minocycline within 0.25 gr of polymer/ mL), empty NPs (PCL, NPs 0.25 gr polymer), free delivered minocycline (mino free, 0.25 mg in addition to PCL) or phosphate

buffered saline, PBS (Saline). Using a glass capillary (40 \pm 2 mm diameter) the solutions (0.250 mL/site) were injected in the injured area of spinal cord with a flow rate of 0.2 mL/min. Six injections were performed in order to cover the whole injured area. The capillary was positioned at \pm 0.5 mm aside from the midline, then it was deepened into the parenchyma to 0.6mm below the pia mater. After treatment, dorsal muscles were juxtaposed using absorbable sutures and the skin sutured and disinfected. During the experiment, mice were housed one per cage with facilitated access to food and water. To compare the efficacy of the intraparenchymal minocycline treatment with the most commonly used intraperitoneal (IP) administration, a group of mice (mino-IP) was treated with intraperitoneal injection of 50 mg/kg minocycline 1 h after surgery. This was followed 24 h later by a second injection of 50 mg/kg. Subsequently, treated mice were injected with a 25 mg/kg dose every 24 h for the next 5 days (Wells et al.; 2003).

3.2 BEHAVIORAL EVALUATIONS All treated mice were evaluated by testing hind-limb locomotor performances using Basso Mouse Scale (BMS) once a week from day 7

e 63 DPI. The BMS is a 10-points scale (9 = normal locomotion; 0 = complete hind limb paralysis). Video acquisition of the locomotor performances (5 min) was performed by camera (ICD-49E, Ikegami) and the evaluation was carried out by two independent observers, blinded to the treatment. Individual hind limb scores were averaged for each animal at each time point.

3.3 TRANSCARDIAL PERFUSION At selected time mice were deeply anesthetized with ketamine hydrochloride (IMALGENE, 100 mg/kg) and medetomidine hydrochloride (DOMITOR, 1 mg/kg) and transcardially perfused with 40 mL of PBS, 0.1 mol/liter, pH 7.4, followed by 50 mL of sodium phosphate buffered 4% paraformaldehyde solution. Spinal cords were rapidly removed, post-fixed for 2 h, transferred to 30% sucrose in PBS at 4°C overnight for cryoprotection and stored at 4°C until use.

3.4 PREPARATION OF SPINAL CORD SECTIONS Spinal cords area around the site of injection (± 5 mm from the epicenter) was embedded in OCT compound and frozen by immersion in N-pentane. Frozen tissues were sectioned in 30 μ m coronal sections on a cryostat at -20°C .

3.5 IMMUNOFLOURESCENCE Immunofluorescence was performed on 30 μ m coronal spinal cord sections. Sections were incubated with different primary antibodies dissolved in PBS, 1% normal goat serum and 0.1% Triton X-100 and incubated overnight at 4°C under constant shaking. Astrocytes were stained with a mouse monoclonal antibody directed to GFAP (Glial Fibrillary Acidic Protein; 1:2500 dilution, Millipore). Microglia were revealed using a rat monoclonal antibody anti-CD11b. To label neuronal cells, spinal cord sections were stained with NeuroTrace Fluorescent Nissl Stain (1:500 dilution, Life technologies, fluorescence excitation/emission maxima 640-660 nm). Oligodendrocytes were detected with MBP (Myelin Basic Protein; 1:100 dilution; Abcam). Sections were then processed with specific secondary antibodies conjugated to Alexa Fluor (1:500 dilution, Life technologies). Finally, spinal cord sections were mounted on slides and coverslipped with a 50% glycerol solution in PBS to proceed with acquisition by confocal microscope.

3.6 IMMUNOFLOURESCENCE QUANTIFICATION

Fluorescent signal was quantified thanks to the free software Fiji (<http://fiji.sc/Downloads>). Single cells were

surrounded with the “free hand” selection and the integrated density of the signal was taken into consideration, as a representative value of both signal intensity and area of the staining. For the evaluation of the cell sphericity, considered an index of microglial cells activation, single cells (about 200) were surrounded, and the “shape descriptors” were calculated through the software Fiji. For each cell, the software gives a value of sphericity ranging from 0 to 1, the last representing a perfect sphere. For the quantification of the CD11b staining in the area around the lesion, a specific plugin of Fiji was used (radial profile plot, <http://rsbweb.nih.gov/ij/plugins/radial-profile.html>). This plugin produces concentric circles around the epicenter of the lesion and gives a graph of the integrated density of the pixel values around each circle. We evaluated up to 100 μm of distance outside the epicenter of the lesion. For the correlation between CD68 expression and cellular uptake of NPs (about 200 cells evaluated), the integrated density of each staining was plotted on a graph, and Person's correlation analysis was taken into account.

3.7 QUANTITATIVE ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA) TNF α , IL-6, IL-1 β and CCL2

concentrations for both cell culture supernatant and homogenate of spinal cord tissues were quantified by solid phase sandwich ELISA (eBioscience, Inc.). Samples from each experiment were tested in triplicate, according to the manufacturer's instructions. To obtain homogenate of spinal cord tissues, at selected time point mice were transcardially perfused with 40 mL of PBS, 0.1 mol/L, pH 7.4. Spinal cords were rapidly removed, frozen on dry ice and then homogenized in 4 volumes of 5X assay diluent provided in the Mouse TNF α ELISA Ready-SET-Go! Kit (eBioscience). For each animal the spinal cord area encompassed the injury or treatment site (6 mm) and a cervical part of the cord (internal control) were removed and analyzed. 96 wells microplates with MaxiSorp surface (NalgeNunc International) were coated with Capture Antibody. After blocking for at least 1 h, 100 μ L of either sample or standard mouse TNF α (provided by ELISA kit) was added to each well and incubated for 2 h at room temperature. After washing, Detection Antibody (200 ng/mL) was added and incubated for 2 h at room temperature. Following the addition of peroxidase substrate solution, the enzyme reaction color product was detected by a microplate

reader set to 450 nm, with wavelength correction set to 540 nm.

3.8 FLOW CYTOMETRIC ANALYSIS At selected time points, B6.129P-Cx3cr1tm1Litt/J mice were deeply anesthetized with ketamine hydrochloride (Imalgene, 100 mg/kg) and medetomidine hydrochloride (Domitor, 1 mg/kg). After a brief perfusion with PBS, spinal cords were rapidly flushed out by the column and the region around the lesion epicenter was passed through a cell strainer (40 μ m-nylon mesh) by pressing with the plunger of a 10 mL syringe, and a cell suspension was obtained by washing the strainer with 5 mL of GKN solution pH 7.4 (8.00 g/L NaCl, 0.40 g/L KCl, 0.93 g/L NaH₂PO₄, 1.42 g/L Na₂HPO₄, 2.00 g/L Dglucose, 0.02% BSA). For each specimen, 1.5 mL of cell suspension was centrifuged at 2000 rpm for 5 min and pellets were resuspended in 500 μ L of FACS blocking (1% BSA and 5% FBS in PBS). After 20 min of incubation in FACS blocking at 4°C, selected primary antibodies (APC-780 conjugated CD11b, eBioscience, 1:100 dilution; APC-conjugated CD45, BD-Biosciences, 1:100 dilution; APC-conjugated Ly6C, BD-Biosciences, 1:400 dilution) were added and incubated for 20 min at 4°C in the dark. One

mL of PBS was then added and specimens were centrifuged at 2000 rpm for 5 min. Pellets were resuspended in 1 mL of FACS blocking before proceeding to the cytometer analysis. In addition, a specific isotype antibody staining was performed as negative control. Immunophenotyping analyses were performed on at least 1.500.000 cells for each sample by using MoFlo Astrios instruments (Beckman Coulter) equipped with 488, 546 and 640 nm lasers. Fluorescence pulses were detected using a band pass filter 531/26 nm for GFP, 576/21 for RhB, 671/20 for CD45 and Ly6C, 795/70 for CD11b. Results were analyzed using Kaluza software (Beckman Coulter). Data are expressed as percentage of positive cells per gated event.

3.9 REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (Real Time RT-PCR) At different time points of analysis, spinal cord regions encompassing the epicenter of the lesion were dissected out, rapidly frozen on dry ice and stored at -80°C until use. Total RNA was obtained from tissues using miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Briefly, spinal cord segments were suspended in QIAzol Lysis Reagent and homogenized with a tissue grinder.

MATERIALS AND METHODS

Chloroform was added to the homogenate and a phase extraction performed. A small volume (0.3 mL) of the aqueous phase was added to 450 mL of ethanol and loaded onto an RNeasy column. The column was washed and RNA eluted following the manufacturer's recommendations. RNA was quantified by a spectrophotometer at 260 nm for all samples. Samples of total RNA were treated with DNase (Applied Biosystems) and reverse-transcribed with random hexamer primers using Multi-Scribe Reverse Transcriptase (Taq-Man Reverse transcription reagents; Applied Biosystems). Realtime RT-PCR was performed using 2 ng of cDNA, 200 nmol of each primer and SYBR Green master mix (Applied Biosystems) in a total volume of 20 mL. Levels of PCR product were measured using SYBR Green fluorescence collected during Real-Time RT-PCR on an Applied Biosystems 7300 system. The expression of the following genes was analysed: YM1 (FW: TCTGGTGAAGGAAATGCGTAAA; REV: GCAGCCTTGGAATGTCTTTCTC); Arg-I (FW: CATGGGCAACCTGTGTCCTT; REV: TCCTGGTACA TCTGGGAACCTTTC); IL1 β (FW: AGTTGACGGACCCCAAAGA; REV: GGACAGCCCAGGTCAAAGG); TNF α (FW:

AGACCCTCACACTCAGATCATCTTC; REV: TTGCTACG
ACGTGGGCTACA); CCL2 (FW: GCCTGCTGTTACAGTTGC;
REV: ATTGGGATC ATCTTGCTGGT); β -Actin (FW:
CGCGAGCACAGCTTCTTT; REV:
GCAGCGATATCGTCATCCAT). β -Actin was used as the
reference gene and relative gene expression levels were
determined according to the manufacturer's DDCT
method (Applied Biosystems). Data are expressed as fold
change from uninjured spinal cord samples (healthy
condition).

3.10 LIGHT SHEET FLUORESCENCE MICROSCOPY (LSFM)

Spinal cords from perfused and fixed mice have been
processed for Light Sheet Fluorescence Microscopy
(LSFM). We performed a whole mount staining of myelin
with green Fluoromyelin (#F34651, Life technologies).
Briefly, specimens were permeabilized through a 4-day-
long procedure. In the first day, specimens were
immersed in Dent's solution (4 parts Methanol, 1 part
DMSO) for 8 h at 4°C and then transferred in Dent's
bleach (1 part H₂O₂ 30%, 2 parts Dent's solution) o.n. at
R.T. Subsequently, spinal cords were placed in methanol
and permeabilized through several passages at -80°C
(45 min at -80°C and then thaw at R.T.). In the third

day, tissues were rehydrated by methanol, treated with Proteinase K (10 µg/mL in PBS) for 5 min and then transferred in a blocking solution containing 0.5% Triton X-100, 4 parts FBS, 1 part DMSO. In the fourth day spinal cords were then transferred in the staining solution containing Fluoromyelin (1:200), 0.5% Triton X-100, 4 parts PBS and 1 part DMSO for 4 days. After the staining, spinal cords were washed in PBS and Triton X-100 0.5% to remove the excess of staining. Then, spinal cords were clarified and imaged through the 3DISCO technique (Ertuk et al.; 2012). Specimens were dehydrated through a crescent series of tetrahydrofuran (THF) (300 in THF 50%, 300 in THF 70%, 300 in THF 80%, 450 in THF 100% x2), lipids were then removed with dichloromethane (DCM) for 200. Tissues were then clarified with a solution of benzyl alcohol and benzyl benzoate (1:1, BABB) and conserved until acquisition in this clearing solution. LSFM was performed using a custom made microscope, whose optical scheme is similar to the system proposed by Dodt et al. (Dodt et al.; 2007). The illumination is provided by two laser beams that are focused with two cylindrical lenses on the sample from two opposite sides, in order to create an illumination plane. A 4x objective (Olympus UPLFLN

4x/0.13 with 17 mm working distance) was used for fluorescence detection. The sample was automatically scanned through the illumination plane while the fluorescence signal was imaged by an Hamamatsu camera (Digital camera ORCA-ER-1394), acquiring about 400 images for each sample. The field of view of the camera was 2.2 mm x 1.6 mm and, in order to image a large volume of the spinal cord, the samples were translated vertically and several regions of interest (typically 7) were acquired. Fluoromyelin Green signal was acquired using a 488 nm laser for excitation, with an emission filter of 525/50 nm (Omega). The regions of interest were stitched by XuvTools software (www.xuvtools.org) and the images were then reconstructed and analyzed using Imaris software (Bitplane). A region of interest (ROI) was applied to measure the fluoromyelin signal of the preserved tissue.

3.11 MICROSCOPY Fluorescent signal was acquired by using a 40X objective magnification, BX81 microscope and Fluoview II camera (Olympus). Colocalization studies were carried out by confocal microscope by using 40X objective magnification, BX81 microscope, FV1000 confocal (Olympus). Time lapse analysis was performed

by using 60X objective magnification, Cell R station (Olympus), ORCA camera (Hamamatsu).

3.12 STATISTICAL ANALYSIS Data were processed using Prism 6 software (Graphpad). Values are reported as mean \pm SEM or mean \pm SD. Mann–Whitney rank sum test was performed to test the significance among groups for *in vitro* NPs uptake assessment, *in vivo* evaluation of CD11B staining at 15DPI, *in vivo* evaluation of CD68 staining at both 3 and 15 DPI, *in vivo* FACS analyses. Kruskal-Wallis and *Post hoc* analysis (Dunn's test) was performed to test the significance among groups for *in vitro* NPs uptake assessment after CPZ treatment and *in vitro* ELISA assay, *in vitro* evaluation of cell sphericity, *in vivo* evaluation of CD11b staining at 3 DPI, whereas a two-way ANOVA and post hoc analysis (Tukey's test) were performed to test the significance among groups for *in vivo* ELISA assay and behavioral analysis. $P < 0.05$ were considered as statistically significant for all analysis.

H. RESULTS

1. INTRODUCTION

Among the physiopathological mechanisms involved in the progression of SCI, inflammation is one of the most relevant. Indeed, recent discoveries demonstrated that acute and chronic inflammation, represented by microglia and cellular infiltration (neutrophils, macrophages and lymphocytes), which subsequently induce the release of inflammatory mediators (cytokines and chemokines), results in the spreading and exacerbation of tissue injury. The strong inflammatory response that occurs after SCI is triggered by the immediate activation of resident microglial cells, able to rapidly respond to insults by changing their own phenotype toward a phagocytic activated shape, and by the subsequent recruitment of monocyte-derived macrophages, that reach the lesion site via the blood stream. Within the lesion, these myeloid cell populations acquire a unique molecular phenotype and share comparable neuromodulatory functions, so in a pathological context they are referred to as microglia/macrophages. Activated microglia and macrophages dominate sites of SCI soon after the trauma and persist in the tissue for several weeks (Sierra et al.; 2013, Loane et al.; 2010). Recently, a dual role (harmful or beneficial) has been associated to different activated microglia/macrophage phenotypes (M1 and M2) during the progression of the traumatic event (Kigerl et al.; 2009, Shechter et al.; 2013). The M1 phenotype is associated with the release of reactive oxidizing species and inflammatory mediators; much

evidence show that the chronic or uncontrolled M1 activation results in neurotoxicity and pro-inflammatory response (Kigerl et al.; 2009, Beck et al.; 2010). Conversely, M2 microglia/macrophages support tissue repair, intrinsic growth and resolution of inflammation. The mechanisms responsible for this functional diversity in microglia/macrophages are not well understood, but are surely conditioned by differential signaling pathways mediated by chemokines and cytokines and other components present in the lesion microenvironment. They appear to be able to activate specific intracellular signaling pathways with opposite results. In a SCI inflammatory context, the dynamic equilibrium of M1/M2 expression has been extensively studied and shows a prevalence of M1 polarization of resident microglia immediately after injury with a subsequent recruitment of M1 macrophages, and an only transient appearance of M2 microglia/macrophages few days after injury. (David and Kroner, 2011; Raposo and Schwartz, 2014; Cohen et al., 2014; Shechter and Schwartz, 2013; Thawer et al., 2013). Unfortunately, this pattern of expression, with a prevalent M1 phenotype acquisition, impairs and limits the recovery and the behavioural outcome after SCI. This suggests that microglia/macrophage associated inflammation could be a persistent deleterious event during the chronic phase of neurodegeneration, representing a crucial therapeutic target for SCI treatment (Cerqueira et al.; 2013, Sato et al.; 2012). In this view, a pharmacological approach specifically tailored to modulate microglia/macrophage activation would provide better chances to interfere with

inflammatory events. Different strategies aimed to selectively modulate microglia/macrophages have already been proposed in preclinical paradigms, such as specific viral carriers, able to transfer genetic sequences and modulate their own expression (Chen et al.; 2005) or various anti-inflammatory treatments (Loane et al.; 2010, Festoff et al.; 2006). However, they showed many limitations. Some safety concerns are associated with gene therapy approaches. Moreover, the short half-life of genes delivered in dividing cells through non-integrative vectors and the possibility that the viral vector is inactivated due to recognition by the immune system, restricts the range of possible applications of gene therapy (Nayerossadat et al.; 2012). On the other hand, anti-inflammatory drugs are not selective for a specific cell type and may give rise to potential side effects associated with systemic administration.

Among the drugs with a direct active effect on microglial cells, minocycline is one of the most promising. In fact, in addition to the well-known antimicrobial activity, minocycline has been reported to have neuroprotective effects in various experimental models such as cerebral ischemia, Amyotrophic Lateral Sclerosis, Parkinson's disease, Huntington disease, Multiple Sclerosis, Traumatic Brain Injury and SCI. Recent studies have demonstrated anti-inflammatory, antiapoptotic and antioxidant properties of minocycline in SCI. Anti-inflammatory effects are mostly associated with the suppression of the proliferation and

activation of the microglial cells and the subsequent release of cytokines such as IL-1 β , TNF α , IL-6, chemokines, and nitric oxide.

However, it has been reported that a prolonged systemic administration of minocycline causes a significant incidence of vestibular effects (dizziness, nausea and vomiting) and pigmentation accumulating in skin and sclera of the eyes (Zemke et al.; 2004).

Recently, a new approach using nanoparticles (NPs)-based tool has been proposed as a selective carrier to deliver compounds directly in microglial cells, namely Qdot nanocrystal (Minami et al.; 2012). This approach exploits the phagocytic activity of activated microglia/macrophages to obtain a selective uptake of NPs by these cell populations. However, a great limitation associated with the capability of these NPs to link chemically to compounds restricts this approach to a scanty number of drugs. As an alternative, polymeric NPs represent a promising pharmacological delivery tool, thanks to their biocompatibility and their ability to sustain the release of drugs. In the first part of this work a new selective delivery tool will be characterized *in vitro*, namely: biodegradable polymeric NPs (100 nm size), obtained through a two-step process, both solvent-free, with a peculiar comb-like structure composed of a poly-(2-hydroxy-ethyl methacrylate) backbone grafted with poly- ϵ -caprolactone (PCL) and PEG chains both controllable in terms of length and composition. These NPs (hereafter termed PCL NPs) present unique properties such as biocompatibility and the possibility to

sustain the release of drugs. Moreover, their high versatility allows control of their degradation rates depending on medical needs.

A selective delivery of minocycline in microglia/macrophages by PCL NPs could enhance drug targeting and concentration at the injury site, reduce side effects, limit drug catabolism and slow down the release of drugs over time.

The selective delivery of drugs through NPs administered *in situ* facilitates also the access of drug to SC. Indeed, conventional drug delivery directed to the damaged spinal cord is limited by the partial physical obstruction due to the BSCB. Most therapeutic compounds are not able to cross the BSCB when administered by oral intake or intravenous infusion. Alternatively, intrathecal delivery by catheters and implanted minipumps has been proposed. However, several disadvantages are associated with the latter delivery techniques, such as side effects of the surgery that is required to implant them and pump refilling.

Accordingly, in the second part of this work the efficacy of an *in situ* minocycline-loaded PCL NPs treatment in reducing the activation of microglia/macrophages in an inflammatory context will be evaluated in an *in vivo* paradigm.

This pharmacological tool will also be useful to understand the real contribution of microglia/macrophage mediated inflammation during SCI progression. Indeed, despite the recent growing effort to characterize

the role associated to the innate immunity response, the specific role played by activated resident microglia during the inflammatory phase remains elusive. Various reasons could explain the difficulties encountered to understand the involvement of activated microglia in this neuropathology: i) microglial cells are extremely complex to target experimentally; ii) their beneficial or detrimental effects are spatially related and temporally defined (Shechter et al.; 2013, Thawer et al.; 2013, Cohen et al.; 2014, Kigerl et al.; 2009); iii) several subsets of microglia are involved (Shechter et al.; 2013, Thawer et al.; 2013, Cohen et al.; 2014, Kigerl et al.; 2009). This raises the importance to understand the functional commitment of inflammatory cells over time and space. Given these critical issues, in the third part of the work an in situ minocycline-loaded PCL NPs treatment will be performed in SCI mice, in order to understand the distinct role played by microglia/macrophage in SCI mouse model and to provide a specific treatment to counteract their deleterious effects during the progression of *secondary injury*.

2. RESULTS

2.1 DEVELOPMENT AND CHARACTERIZATION OF A MICROGLIA/MACROPHAGE TARGETED NANOVECTOR *IN VITRO*

2.1.1 Aims

In order to increase the efficacy of potential drugs in modulating microglia/macrophage mediated inflammation after SCI, the aim of the first part of this work is to develop and characterize *in vitro* a new polymeric NPs drug delivery system for the selective pharmacological treatment of activated microglia/macrophage.

In particular, the distribution and biocompatibility of polymeric NPs composed of poly-(2-hydroxy-ethyl methacrylate) and poly- ϵ -caprolactone (PCL), conjugated to Rhodamine B (RhB), will be evaluated in *in vitro* co-cultures of microglia, astrocytes and neurons.

2.1.2 Evaluation of selective microglia/macrophage uptake of PCL nanoparticles

Primary co-cultures of microglia, astrocytes and neurons obtained from the spinal cord of mouse embryos (see methods) were used to characterize the uptake of PCL NPs previously described. Specific antibodies were used to detect astrocytes, microglia and neurons in co-culture (respectively stained for GFAP, CD11b and NF200). In order to mimic an inflammatory environment, cell cultures have been treated for 18 hours with LPS, a potent immuno-activator of monocytes and macrophages, widely used to activate microglia cells *in vitro*. Morphological changes have been verified for LPS-stimulated microglial cells, which showed an activated hypertrophic shape (amoeboid/phagocytic shape) in comparison to untreated microglial cells used as the control condition (Fig. 1.1, A). No relevant morphological alterations were found for astrocytes and neuronal cells (Fig. 1.1, A) after LPS stimulus. In addition, a cell viability assay in neuroglial culture was performed showing a lack of both morphological alteration and viability in astrocytes, whereas 30% of neuronal death was detected (data not shown). The NPs uptake evaluation was performed only in healthy neuroglial cells detected as negative staining for Propidium Iodide (cell death marker). Co-cultures were exposed to PCL NPs for 48h (1:100 dilution rate) (Fig. 1.1 A). A selective internalization in microglial cells of a few NPs has been observed in

unstimulated cultures (Fig. 1.1 A, a,b). Noteworthy, a remarkable increased uptake of PCL NPs was detected in LPS-stimulated microglial cells (Fig. 1 A, c,d) compared to unstimulated condition, as shown by a higher diffuse RhB signal in the cytosol of activated microglia. Quantification of RhB internalized in microglia after PCL NPs treatment confirms this observation, revealing a significant difference between unstimulated and LPS stimulated microglia (about 1.7 fold) (Fig. 1.1, B).

The selectivity of PCL NPs uptake by microglial cells was further confirmed by the absence of colocalization between red signal of RhB and blue signal of NF200 (Neurons, Fig. 1 A, a,c) or GFAP (Astrocytes, Fig. 1 A, b,d).

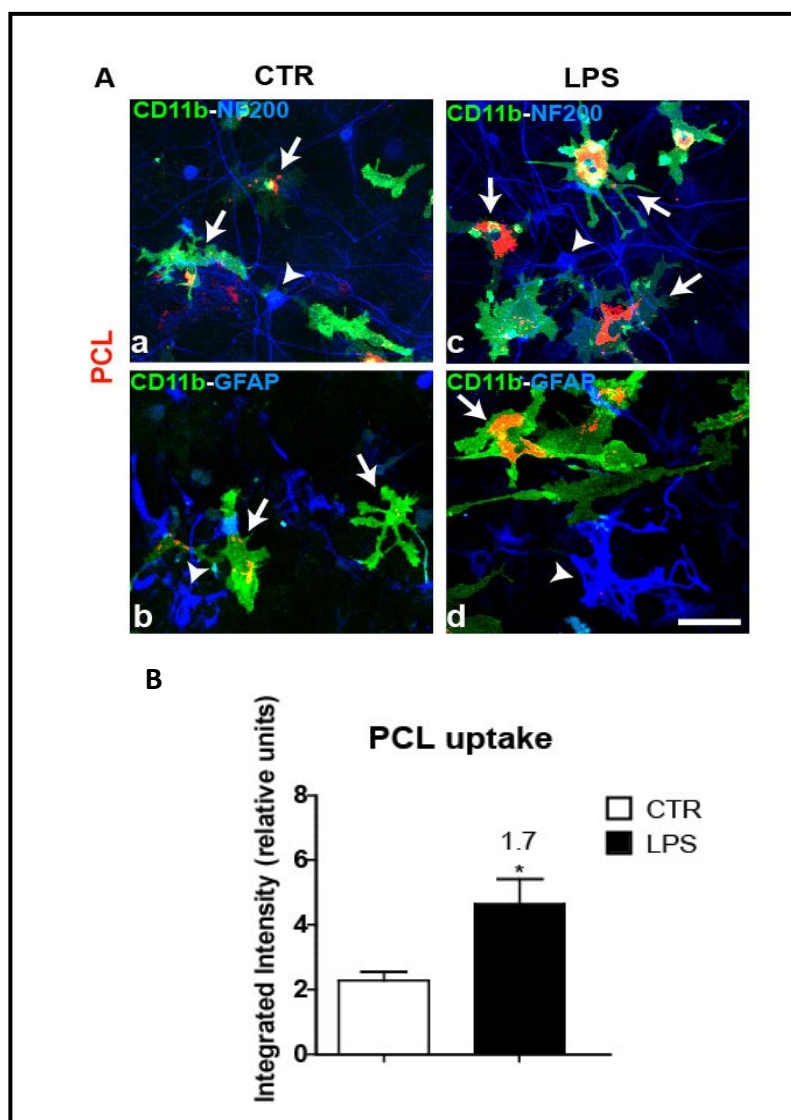


Figure 1.1. *In vitro* characterization of PCL NPs uptake, in primary co-cultures of microglia, astrocytes and neurons. Accumulation of fluorescent PCL NPs (**A**) is markedly increased in LPS stimulated microglia (c,d arrows) compared to control condition (a,b arrows). No uptake is observed for both neurons (a,c arrowheads) and astrocytes (b,d arrowheads). Microglia, CD11b (green); NPs (RhB), (red); neurons, NF200 (blue) (a,c); astrocytes, GFAP (blue) (b,d). Scale bar=15 μ m. Uptake quantification of PCL NPs (**B**) in activated microglia (LPS) vs unstimulated cells (CTR). Data are presented as mean \pm SEM. Statistical significance: (*) $p \leq 0.05$.

2.1.3 Time lapse analysis of PCL NPs uptake

In order to investigate the PCL NPs uptake kinetics, time-lapse analysis was performed on purified cultures of LPS-stimulated microglia. Time-lapse analysis after PCL NPs uptake showed that NPs uptake was detectable already after 24 hours of treatment, reaching a plateau after 48 hours of incubation (Fig. 1.2 A). After internalization, a cytosolic localization of the RhB signal was found in activated microglia, progressively evolving in a cytosol-perinuclear intense distribution, as revealed at the end of the acquisition (Fig. 1.2 A).

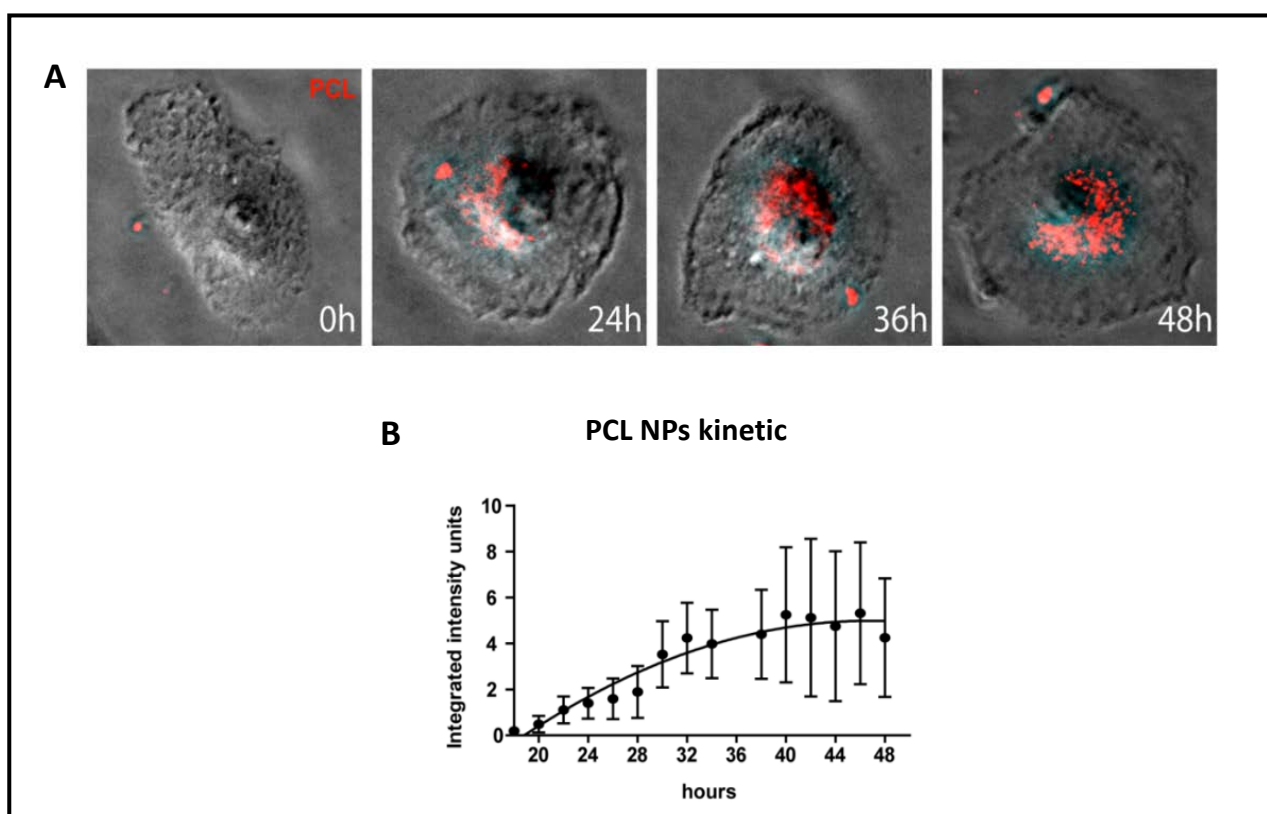


Figure 1.2. Time-lapse analysis of PCL NPs uptake kinetics. (A) RhB red signal shows a perinuclear distribution after 48 hours of treatment. (B)

Quantitative evaluation of progressive PCL NPs internalization in activated microglial cells during time lapse analysis. Data are presented as mean \pm SEM.

2.1.4 Evaluation of cell viability after PCL NPs uptake

To evaluate the biocompatibility of PCL NPs after internalization, we tested the cell viability in purified LPS-stimulated microglia cultures. PCL NPs uptake did not affect microglial cell viability up to 6 days, as detected by MTT assay comparing LPS-stimulated cells treated or not with PCL NPs (Fig. 1.3 A). Furthermore, we assessed the release of three inflammatory cytokines (IL-1 β , IL-6 and TNF α) in co-cultures, after treatment with NPs, in order to evaluate whether PCL NPs could be a further stimulus to release pro-inflammatory cytokines. We removed the medium after 18 hours of LPS stimulation and we treated cells with NPs for 48 hours (uptake plateau for PCL), detecting the subsequent release of pro-inflammatory cytokines from activated microglia. Analyzing the medium for IL-1 β , TNF α and IL-6 by ELISA assay, we found that IL-1 β and TNF α were below the detection limit, whereas IL-6 showed a significantly increased level in the medium of LPS stimulated co-cultures (148.6 \pm 10.70 mean pg/ml \pm SD) compared to unstimulated cultures (66.8 \pm 35.3 mean pg/ml \pm SD) (Fig. 1.3 B). When we analyzed LPS-stimulated microglial cells exposed to NPs, no significant differences in the level of IL-6 were detected (169.8 \pm 8.5 mean pg/ml \pm SD) compared to LPS-stimulated co-cultures.

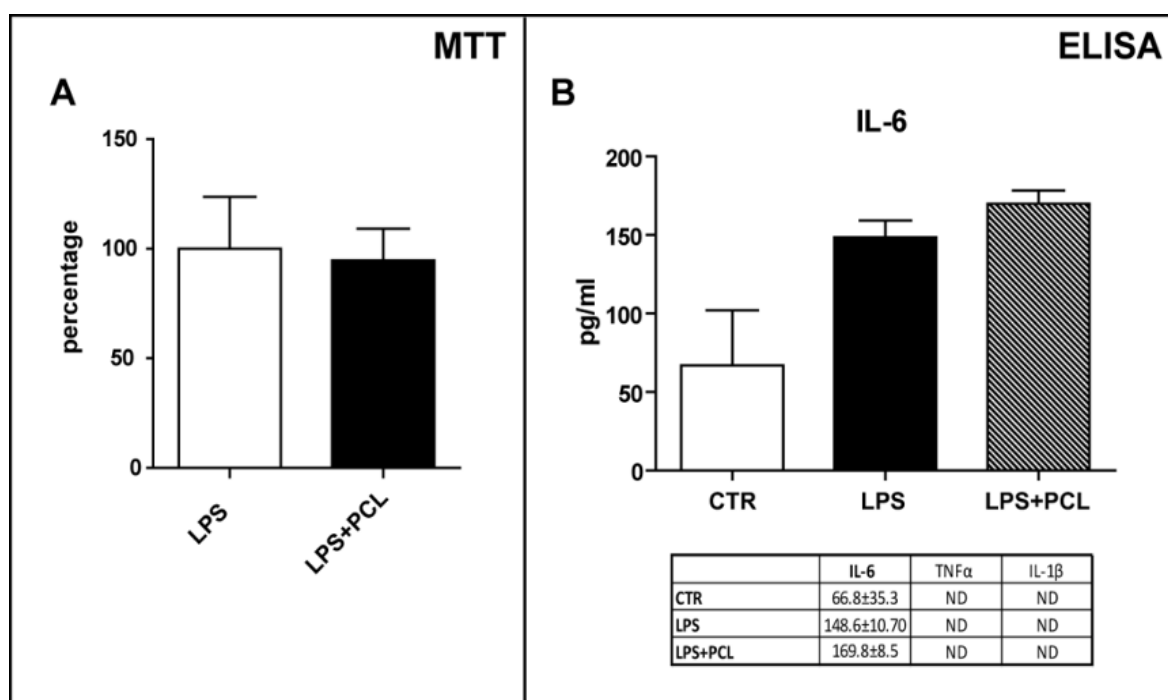


Figure 1.3. Cell viability and ELISA assay of microglial cells cultures after internalization of PCL NPs. **(A)** MTT assay reveals that viability of LPS stimulated microglial cells after 6 days of treatment with PCL NPs is not compromised. Data are presented as percentage \pm SD of viable cells normalized to LPS values (no particles exposure), which represents 100% of viability. **(B)** ELISA assay performed on the supernatants of LPS stimulated cells shows that after treatment with PCL NPs, the release of pro inflammatory cytokine IL-6 is not significantly increased. Data are presented as mean \pm SD.

2.1.5 Evaluation of PCL NPs uptake mechanism

In order to understand the mechanism underlying PCL NPs internalization, we tested the effect of chlorpromazine (CPZ), a clathrin-mediated endocytosis inhibitor. Different concentrations of CPZ (30-40 μ M) were used in LPS stimulated co-cultures as pre-treatment for 2 hours, before exposure to NPs. Quantification of RhB signal in

stimulated microglia revealed that a significant dose-dependent inhibition of PCL NPs uptake was induced by 30 (2.15 ± 0.39 mean integrated intensity \pm SEM) and 40 μ M (1.21 ± 0.26 mean integrated intensity \pm SEM) of CPZ (Fig. 1.4 A), compared to cells not pretreated with CPZ (LPS), used as control condition (4.90 ± 0.64 mean integrated intensity \pm SEM) (Fig. 1.4 A,B). These data suggest that clathrin-mediated endocytosis has a determinant role on PCL NPs uptake in activated microglia.

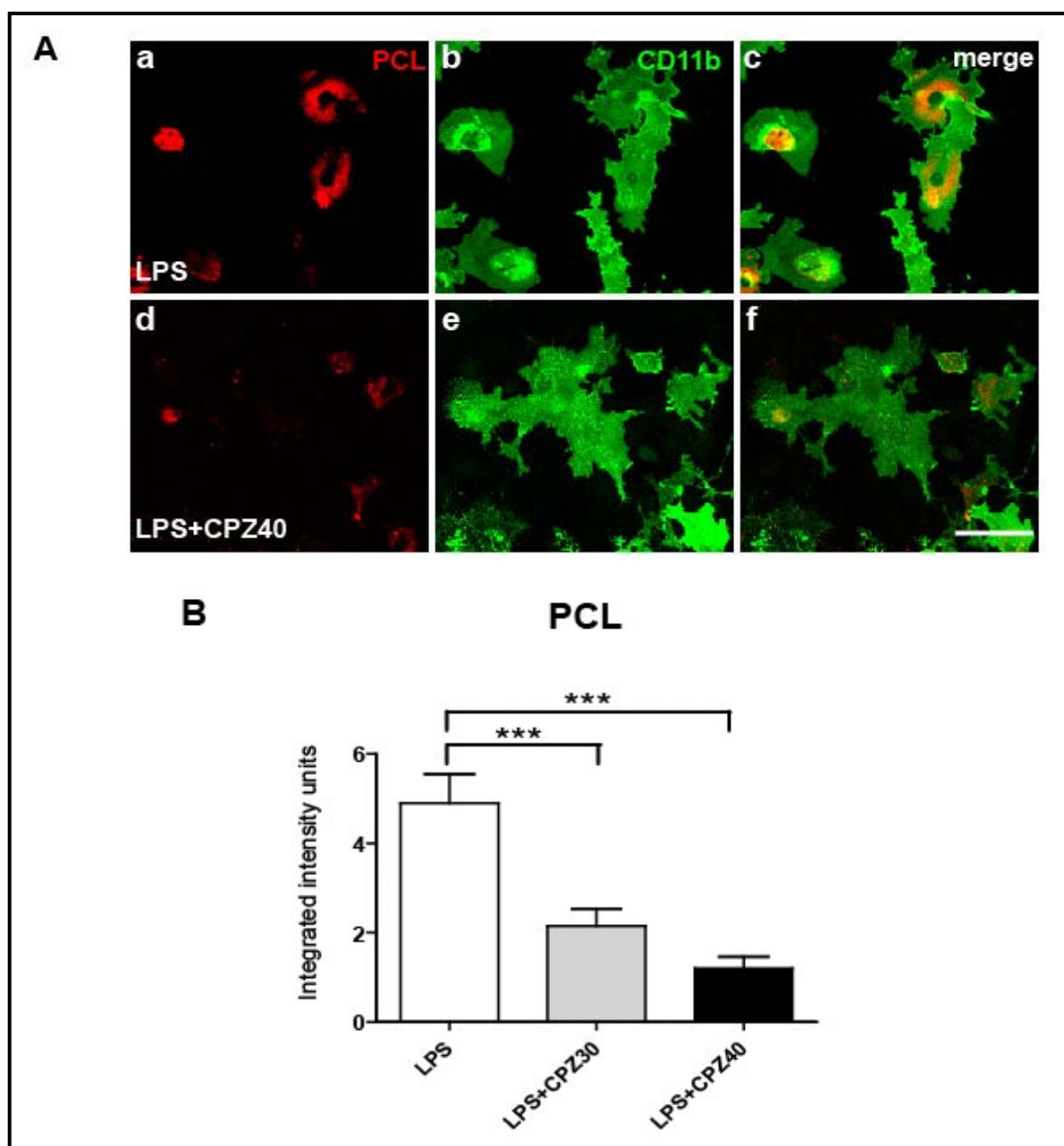


Figure 1.4. (A) Pre-treatment with CPZ (a-f) is able to inhibit PCL NPs uptake in activated microglia *in vitro*. Microglia, CD11b (green); PCL, (RhB) (red). Scale bar=5 μ m **(B)** Quantitative analysis of PCL NPs uptake shows that uptake inhibition occurs in a dose-dependent manner. Data are presented as mean \pm SD. Statistical significance: (***) $p\leq 0.001$.

2.1.6 Evaluation of PCL NPs intracellular distribution and degradation

In order to investigate the intracellular distribution of NPs, further analyses using transmission electron microscopy (TEM) have been carried out after the internalization of NPs in activated microglial cells. We used not biodegradable- polymeric NPs composed of poly-methyl-metacrilate (PMMA) as control treatment for this experiment. PCL and PMMA NPs were visible by TEM as white dots (Fig. 1.5 A, a) and after internalization were stored in a well-defined structure, identified as multivesicular bodies (MVBs). In spite of a large amount of NPs internalized, no other morphological alterations have been detected in the organelles of the cytosol. In fact, a normal structure of mitochondria, nucleus, endoplasmic reticulum and Golgi apparatus was found in activated microglial cells exposed to NPs, compared to microglial cells not exposed to NPs (Fig. 1.5 A, b), confirming the biocompatibility of both polymeric NPs. Two days after PCL NPs treatment, we observed areas of PCL NPs debris in the intraluminal vesicles of MVB digested by the fusion with lysosomes (Fig. 1.5 B, a,b). At 6 days after treatment a complete degradation of PCL NPs was found, as shown by more diffuse polymeric debris in lysosomes (Fig. 1.5 B, c,d, arrows). Conversely, PMMA NPs showed an identical white spherical shape in MVBs, at both 1 and 6 days after microglial cells treatment (Fig. 1.5 C, a-d, arrows). However, at 6 days after treatment

NPs resulted more disperse in both cytosol and MVBs (Fig. 1.5 C, c,d). In order to verify the lysosomal enzymatic activity after PCL and PMMA NPs internalization, a lysosensor, a fluorescent pH indicator that measures the activity of acidic organelles, was used to study the lysosome function in live cells after NPs treatment. The analysis revealed an increased lysosomal enzymatic activity, represented by an increased green signal highly colocalized with PCL RhB red signal, at 6 days after PCL NPs treatment (Fig. 1.5 D, a). This result confirms the biodegradability of PCL, which are rapidly sequestered by MVBs at an early stage and then completely degraded by lysosomes.

Conversely, only some green lysosomal spots in the cytosol of microglial cells treated with PMMA NPs with a lack of colocalization with RhB signal, suggesting a lack of degradation of PMMA NPs by microglia (Fig. 1.5 D, b). These results underline the biodegradability of PCL NPs, compared to PMMA NPs.

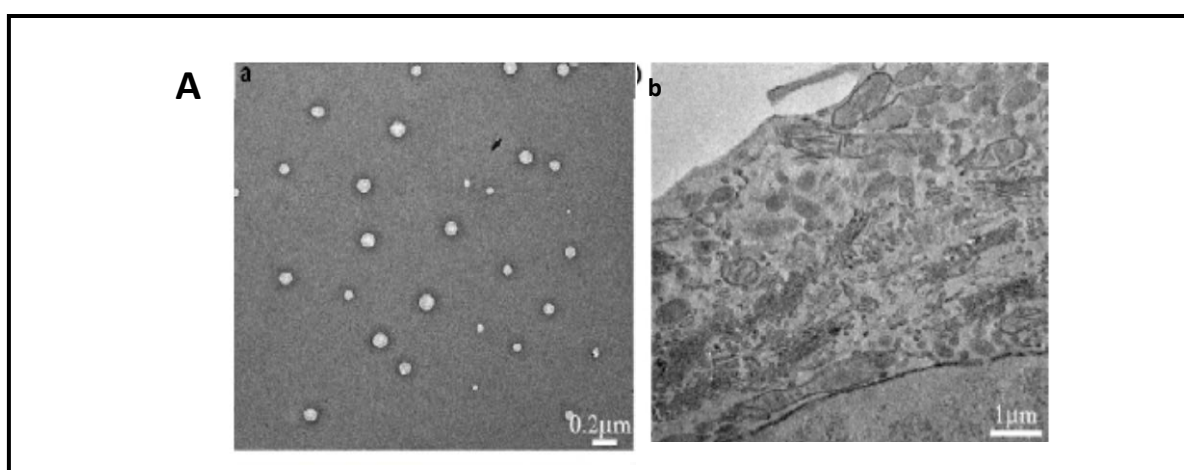


Figure 1.5. (A, a) Representative single PMMA NPs in TEM analysis. (b) Organelles of the cytosol don't show any morphological alteration after PCL or control (PMMA) NPs treatment.

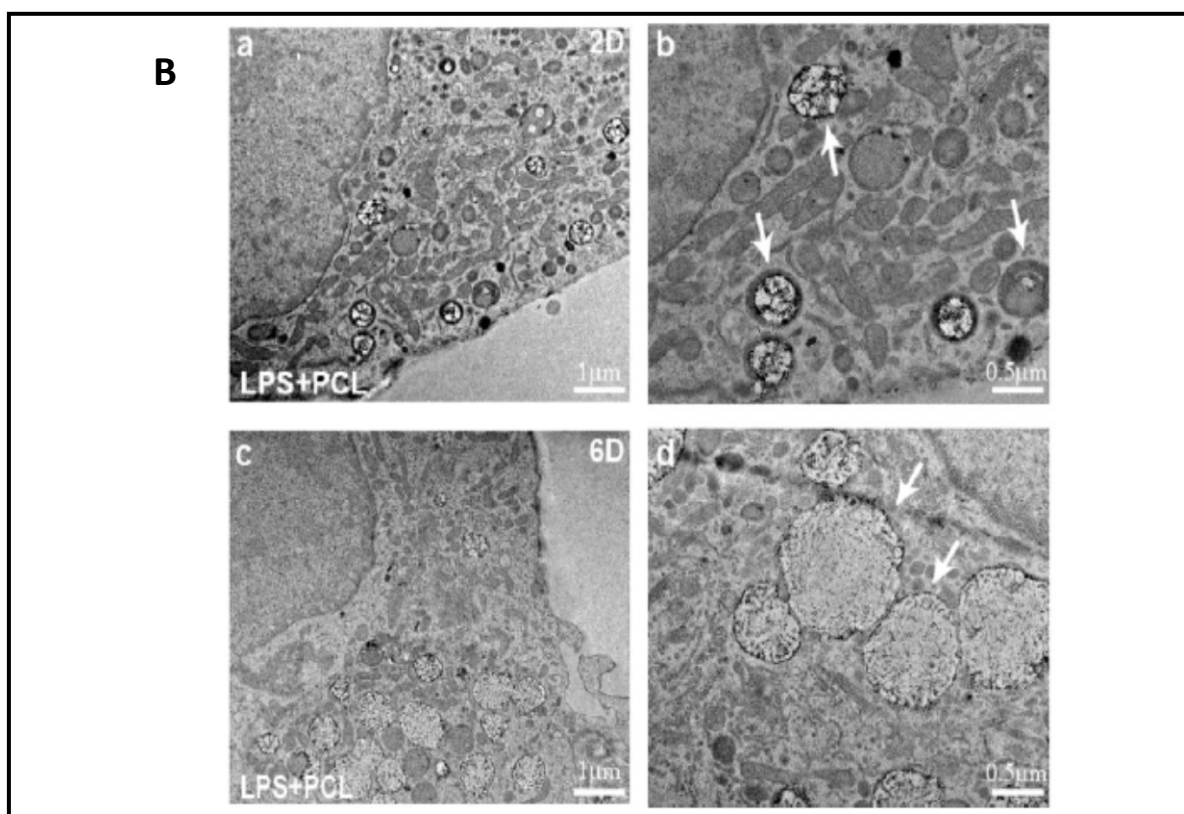


Figure 1.5. (B) TEM analysis of activated microglia 2 (2D, a, b) or 6 days (6D, c, d) after treatment with PCL NPs. NPs are taken up and stored in MVBs in the cytosol of activated microglia (b, arrows) and are degraded after 6 days (d, arrows).

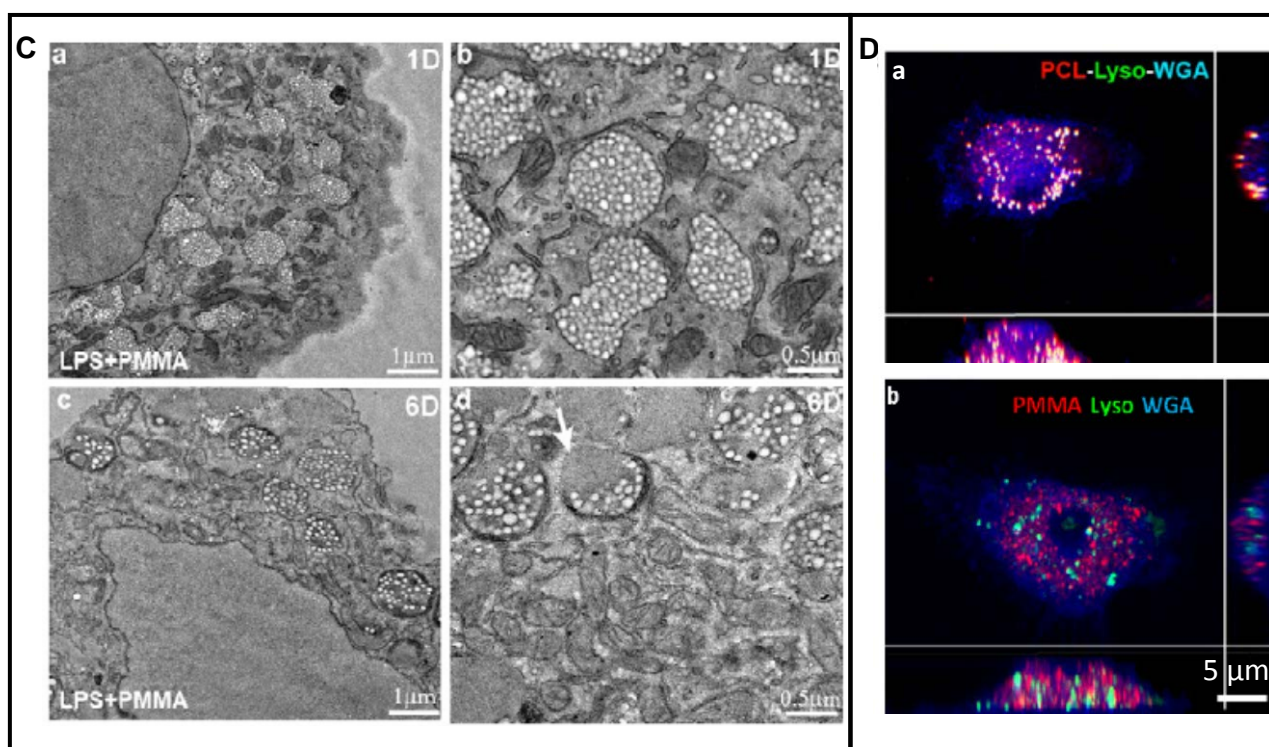


Figure 1.5 (C) TEM analysis of activated microglia 1 (1D, **a**, **b**) or 6 days (6D, **c**, **d**) after treatment with PMMA NPs. NPs are taken up and stored in MVBs in the cytosol of activated microglia without a clear evidence of degradation. (D) Lysosomal activity after PCL (**a**) or PMMA (**b**) NPs internalization. Orthogonal projection of confocal image stack shows that red signal of PMMA does not colocalize with lysensor marker, while an increased lysosomal enzymatic activity was measured after PCL treatment. PCL or PMMA NPs, RhB (red); lysosomes, lysosensor (green); cell membrane, Alexa Fluor®350 WGA (blue).

2.1.7 Discussion and Conclusions

In this part of the work a new pharmacological approach has been validated, based on biodegradable polymeric NPs, that is able to selectively target activated microglial cells *in vitro*. This may represent a

potential tool able to modulate selectively microglia/macrophages activation in SCI.

In particular, it has been demonstrated *in vitro* that: I) PCL NPs are internalized selectively by microglial cells in a short time (some hours); II) microglia in activated form are able to internalize markedly PCL NPs, whereas microglia displaying a resting phenotype are not; III) PCL NPs show a complete biocompatibility and degradation when internalized in activated microglial cells; IV) PCL NPs internalization in activated microglial cells is based on clathrin-dependent endocytosis mechanisms; VI) PCL NPs do not induce further proinflammatory stimuli or any toxicity. The selective and specific pharmacological tool here characterized could provide, compared to the conventional anti-inflammatory administration tools, some potential advantages for its use in *in vivo* SCI context: I) an improved efficacy of treatment by increasing the concentration of the drug in specific target cells in the site of injury; II) reduced potential side effects due to the possibility to avoid unwanted distribution of the drug to not-target cells or tissues; III) time-selective treatment of microglial cells offers the potential to modulate the activation of the microglial cells at different stages of the secondary injury and the possibility to deepen the understanding of the detrimental or beneficial role of the microglia during the progression of the disease.

2.2 Characterization of PCL NPs cellular uptake and evaluation of the pharmacological activity of minocycline loaded PCL NPs *in vivo*

2.2.1 Aims

PCL NPs are biocompatible and biodegradable polymeric nanoparticles that are selectively taken up by activated microglia after an inflammatory stimulus *in vitro*. PCL NPs are able to load drugs and after cellular uptake they are able to release their content, allowing a targeted therapy to microglia/macrophage, key players in the inflammatory cascade. The aim is to evaluate in an *in vivo* paradigm the cellular distribution of PCL NPs in the CNS in an inflammatory milieu and to characterize *in vitro* and *in vivo* the pharmacological activity of minocycline, a well-known anti-inflammatory drug, loaded into PCL NPs, and able to modulate selectively the microglia/macrophages activation.

2.2.2 Cellular uptake of polymeric PCL NPs *in vivo*

To evaluate and characterize the distribution of PCL NPs *in vivo*, an injection of hydrogel loaded with PCL NPs was performed within the parenchyma of mouse SC in order to induce an acute injury and an inflammatory response, as described in materials and methods section and previously published by our lab (Perale 2011). Early after the injection, the hydrogel revealed a spherical enclosure, which physically

displaced the tissue in the SC, due to its in situ gelation. To investigate the inflammatory response of the nervous tissue to injury induced by the injected hydrogel, a double staining with markers for microglia (CD11b) and astrocytes (GFAP) or neurons (Neurotrace) was carried out. At 3 days post injection (DPI), an inflammatory reaction was detected around the hydrogel, identified as a glial scar formation composed of phagocytic/ameboid microglia and astrocytes (Figure 2.1 A).

A distribution of PCL NPs was found around the injected hydrogel, revealing a selective uptake of NPs into the nearest activated-phagocytic microglia/ macrophages (CD11b) (Figure 2.1 B). The selective uptake by microglia/macrophages was confirmed detecting a lack of colocalization between NPs (RhB) and GFAP/Neurotrace which identifies respectively astrocytes and neurons (Figure 2.1 C, a,b).

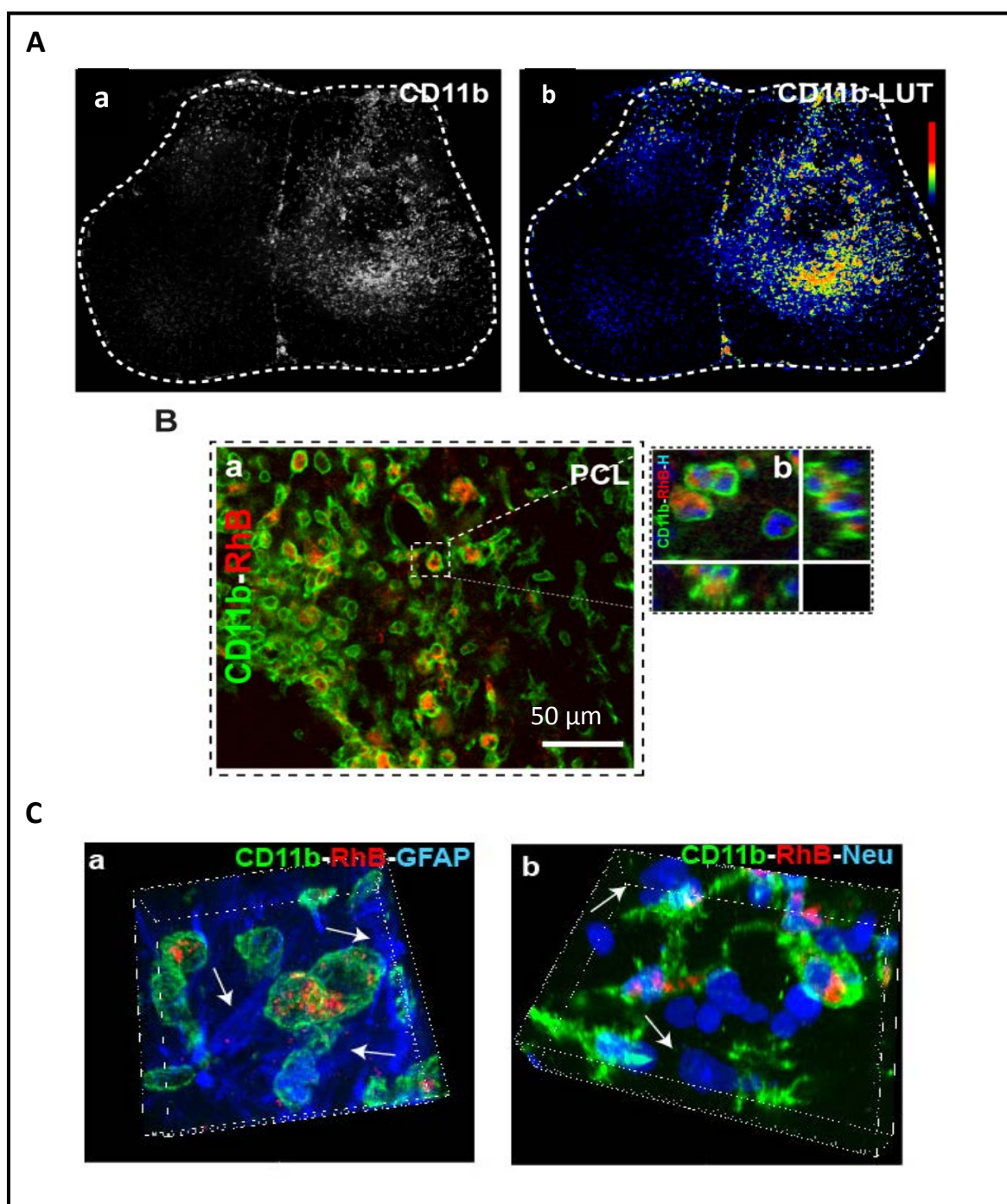


Figure 2.1. (A) Dashed lines outline representative low magnification coronal sections of mouse spinal cord injected, three days before, with PCL NPs loaded hydrogel. CD11b staining shows the proliferation/activation of microglia/macrophages around injection site. (a) Microglia, CD11b and (b) its representation in pseudocolors. Scale bar= 50 μm. (B) (a). High magnification of spinal cord section of a mouse injected with PCL NPs loaded hydrogel and (b) relative inset representing three-dimensional details of activated

microglia/macrophages and PCL NPs uptake around the injection site **C**. High magnification of selective PCL NPs uptake by microglia/macrophages *in vivo* without a clear evident uptake in astrocytes (**a**, arrows) or neuronal cells (**b**, arrows).

2.2.3 Study of the pharmacological activity of minocycline selectively delivered in microglia/macrophages by PCL NPs *in vitro*

Minocycline was loaded in preformed PCL NPs (PCL Mino) by entrapment of the hydrophobic drug in partially swollen NPs, followed by 4 h dialysis to remove unloaded drug, as assessed by HPLC_MS/MS (see materials and methods section for details). At this dialysis time point, PCL NPs retain about 35% of the added minocycline, with a final loading of about 1 µg of minocycline/mg of NPs. Microglial cells *in vitro* were analyzed for their morphological criteria by comparing (I) unstimulated control cells (CTR); (II) LPS-stimulated cells (LPS); (III) LPS stimulated cells treated with free minocycline (LPS+Mino, 0.07 µg/mL); (IV) LPS-stimulated cells subsequently exposed to PCL (LPS+PCL); (V) LPS-stimulated cells subsequently exposed to both PCL and free minocycline (LPS+PCL+Mino, 0.07 µg/mL); and (VI) LPS-stimulated cells treated with minocycline encapsulated in PCL NPs (LPS+PCL Mino, 0.07 µg/mL loaded). Unstimulated microglial cells showed a small soma with some ramified processes (Figure 2.2 A, a). Differently, LPS-stimulated microglial cells displayed a round shape typical of fully activated microglia with a macrophage phenotype (Figure

2.2 A, b). Evaluating activated microglial cells treated for 96h with PCL Mino, a relevant regression toward a quiescent phenotype was found (Figure 2.2 A, d). In fact, a reduced round shape phenotype and the reappearance of thick processes could be appreciated after PCL Mino treatment. This was confirmed also by quantitative evaluation of the sphericity (from 0 to 1, respectively, ramified/oval and round shape) as a morphological parameter for the evaluation of the activation state of the microglial cells. This assessment showed a significantly reduced value for LPS+PCL Mino (0.31 ± 0.13 mean sphericity \pm SD) compared to LPS+PCL (0.67 ± 0.17 mean sphericity \pm SD) (Figure 2.2 C). The regression toward a more quiescent phenotype was further demonstrated by time lapse analysis on single cells that showed a morphologically defined stepwise deactivation up to 96 h (Figure 2.2 B). Analyzing LPS+PCL (0.67 ± 0.17 mean sphericity \pm SD) an increased, but not significant, sphericity was detected compared to LPS (0.52 ± 0.09 mean sphericity \pm SD), likely due to NPs uptake (Figure 2.2 C). A significant reduced sphericity was detected also for cells treated with LPS+Mino (0.41 ± 0.12 mean sphericity \pm SD) and LPS+PCL+Mino (0.52 ± 0.10 mean sphericity \pm SD) when compared respectively to LPS (0.51 ± 0.09 mean sphericity \pm SD) and LPS+PCL (0.67 ± 0.17 mean sphericity \pm SD) stimulated cells (Figure 2.2 A, C), but with a significantly lower efficacy compared to activated microglia treated with PCL Mino (0.31 ± 0.13 mean sphericity \pm SD). All groups showed an increased significant sphericity compared to CTR group (LPS, LPS+Mino,

LPS+PCL, LPS+PCL+Mino $p \leq 0.0001$ (****) and LPS+PCL Mino $p \leq 0.05$ (*).

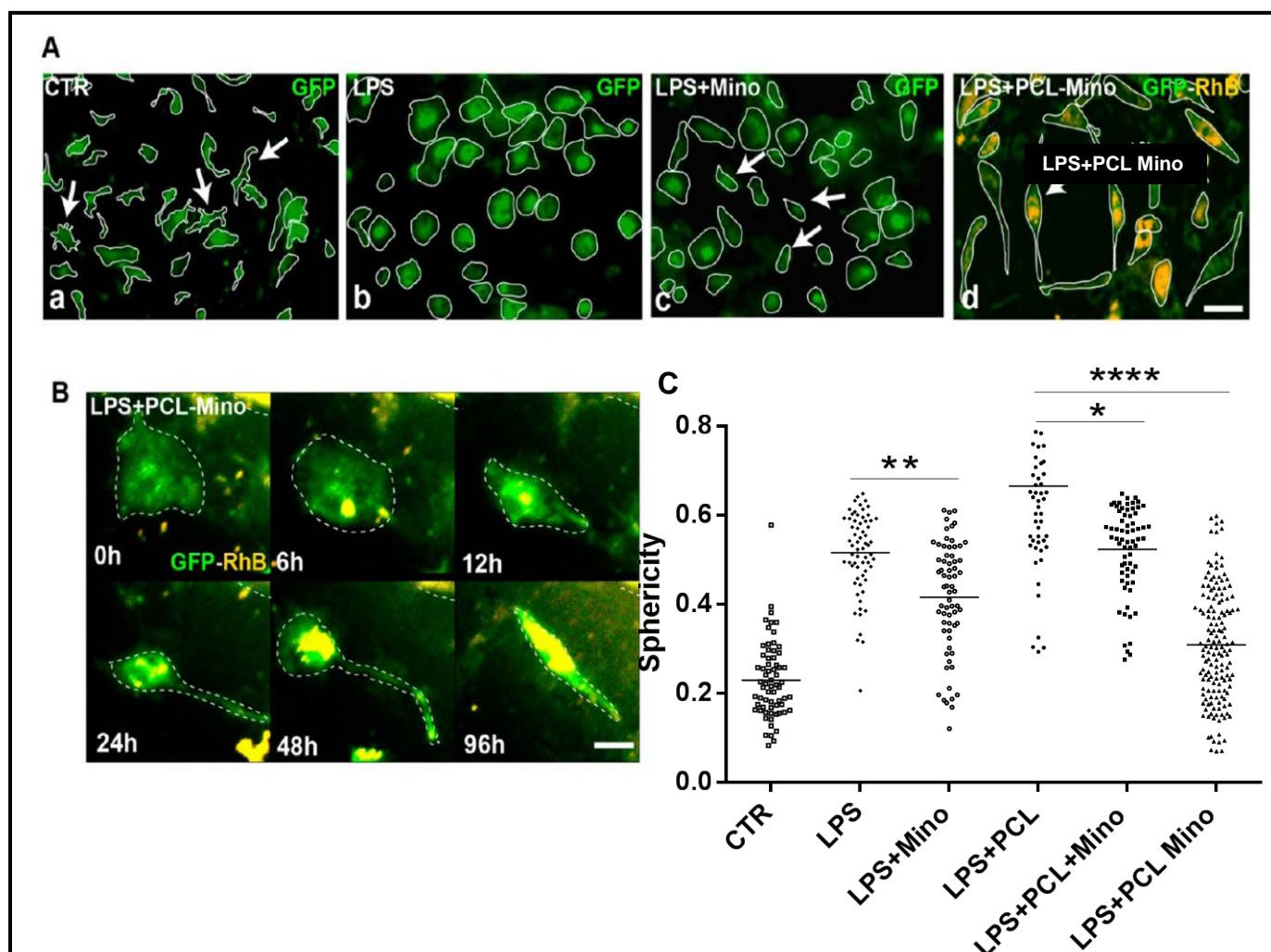


Figure 2.2. PCL Mino treatment of activated microglia is able to revert activation process *in vitro*. **(A)** In primary GFP microglia cultures from CX3CR1 mouse embryos, LPS treated cells **(b)** were incubated with PCL (LPS+PCL) **(c)** or with PCL Mino (LPS+PCL-Mino) and analyzed 96 h later **(d)**. Scale bar=10 μm . **(B)** Time lapse imaging of a single microglial cell during 96 h of treatment with LPS+PCL Mino. At the end of the analysis, microglia show a more ramified phenotype. Microglia, GFP (green); NPs, PCL Mino RhB (orange). Scale bar=5 μm . **(C)** Evaluation of cell sphericity (index of cells activation) of microglial cells after five different treatments. Sphericity of LPS+PCL Mino treated cells is

significantly lower than either LPS+PCL or LPS+PCL+Mino treatment. Each dot represents the sphericity of a single cell. Statistical significance: (*) $p \leq 0.05$; (**) $p \leq 0.01$; (****) $p \leq 0.0001$.

2.2.4 Study of the pharmacological activity of minocycline selectively delivered in microglia/macrophages by PCL NPs *in vivo*

To determine the potential pharmacological activity of drugs delivered by PCL NPs previously described, we tested biodegradable PCL NPs loaded with minocycline (PCL Mino) *in vivo*. To do that, an injury induced by injected hydrogel was carried out in adult mice (as described in materials and methods section). We compared mice injected with hydrogel and unloaded PCL NPs (PCL), as control, hydrogel and free minocycline (Mino, 35 $\mu\text{g/mL}$) and hydrogel and minocycline encapsulated in PCL NPs (PCL Mino, 35 $\mu\text{g/mL}$ loaded) at two different time points, 3 and 15 DPI. *Ex vivo* analysis revealed a reduced proliferation of microglia/macrophages around the injured site in Mino mice at 3 DPI (Figure 2.3 A, B, C). Indeed, the quantification of CD11b staining around the injection site, detected up to 100 μm from the center of the lesion, revealed that Mino treated mice had a significant reduction (931 ± 283 mean integrated intensity (SD) compared to control mice (PCL) (1630 ± 241 mean integrated intensity (SD) (Figure 2.3 C)). However, the activated microglia/macrophages around the

injection site in Mino treated mice still showed a round shape, suggesting a likely reduced proliferation of these cells without a clear morphological changing to quiescent phenotype.

Analyzing PCL Mino mice at 3 DPI, a significant reduction of CD11b staining was detected, suggesting a decreased proliferation and activation of microglia/ macrophages (767 ± 237 mean integrated intensity (SD) (Figure 2.3 A, C). Moreover, an evident morphological change toward a more quiescent phenotype (ameboid/resting shape) was found in RhB positive microglia/macrophages around the injection site (Figure 2.3 A, B) compared to both PCL and Mino mice.

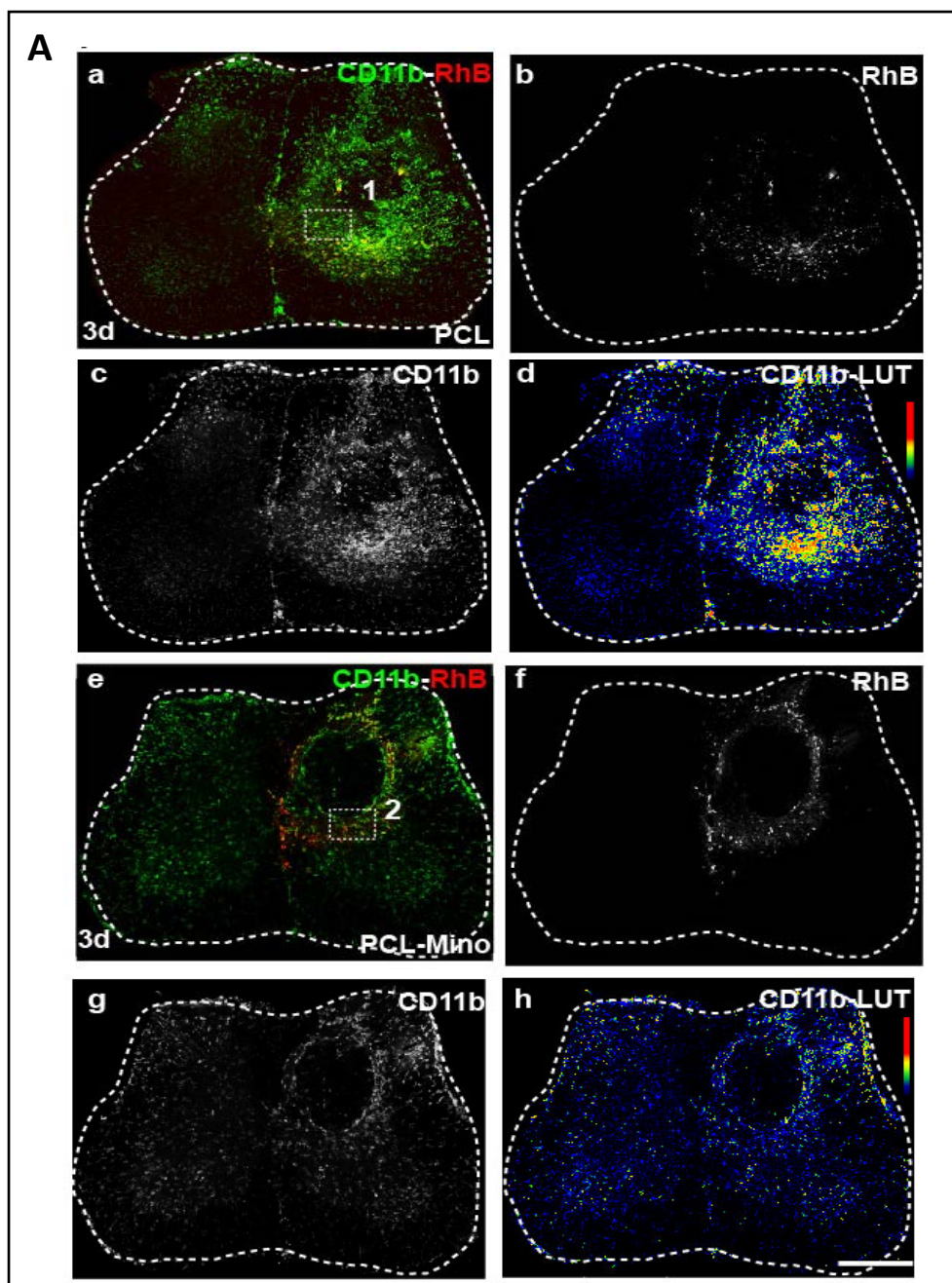


Figure 2.3 (A) PCL Mino treatment of activated microglia/macrophages is able to revert activation process induced *in vivo* by hydrogel injection. Dashed lines outline representative low magnification coronal sections of mouse spinal cord injected, three days before, with PCL loaded hydrogel (a_d, PCL) or PCL Mino loaded hydrogel (e_h, PCL-Mino). CD11b staining in PCL Mino treated mice shows a reduced proliferation/activation of microglia/macrophages around the site of injection compared to PCL mice. Microglia, CD11b (green); PCL NPs, RhB (red); CD11b staining pseudocolors image (d_h). Scale bar 150 μm.

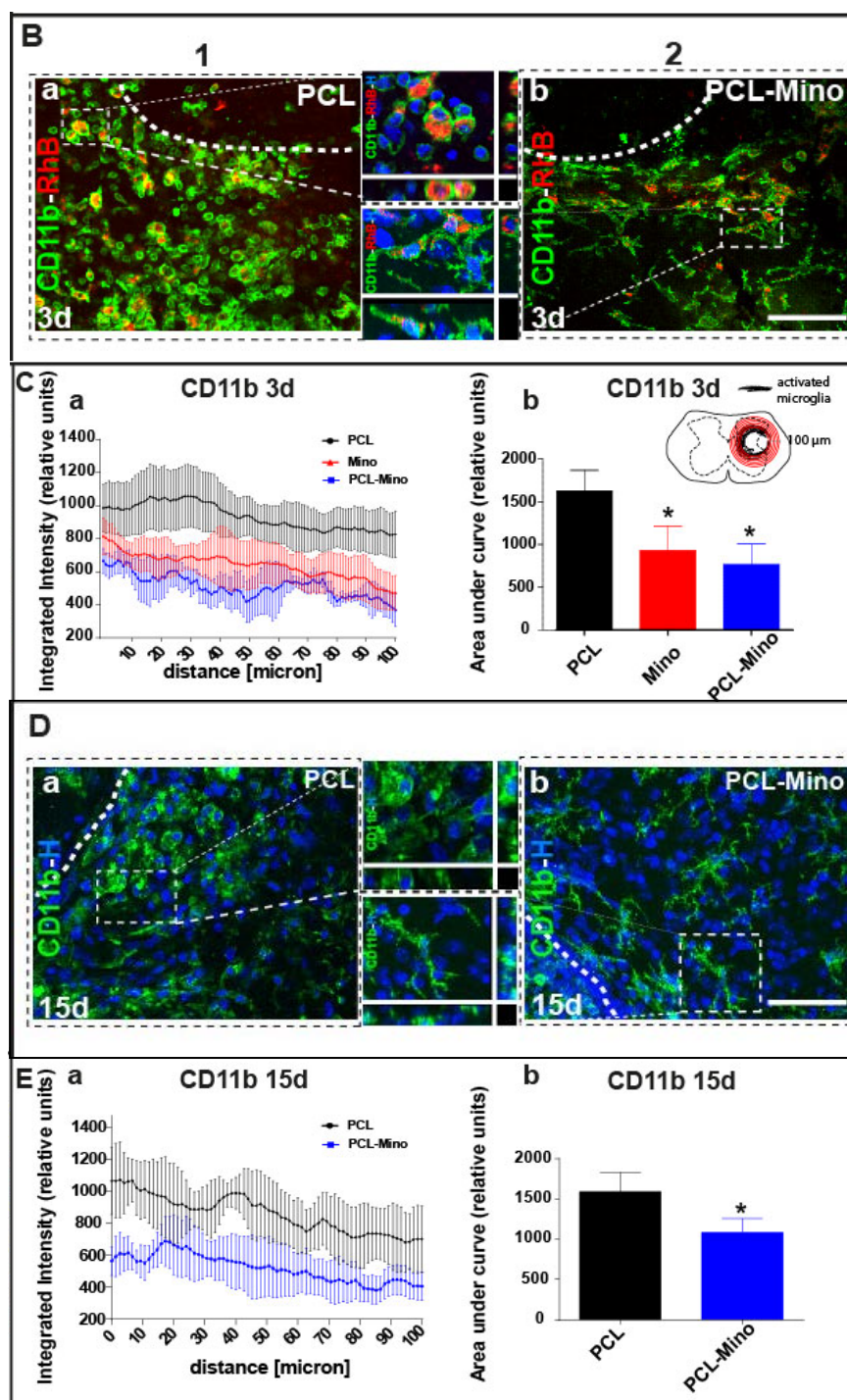


Figure 2.3 (B, D) High magnification of spinal cord sections of a mouse treated with PCL NPs **(B;a)** or with PCL Mino NPs **(B;b)** representing, respectively, region 1 and 2 identified by dashed line in panel A. Insets represent three-dimensional details of microglia/macrophages around the lesion site in both PCL and PCL Mino NPs. PCL Mino NPs induce an evident morphological change toward a quiescent phenotype in CD11b positive microglia/macrophages that have taken up PCL Mino NPs compared to PCL

treatment, and this deactivation is evident up to 15 DPI (**D; a,b**). Microglia, CD11b (green); PCL or PCL Mino, RhB (red); cell nuclei, Hoechst (blue). Scale bar=50 μ m. (**C, E**) Evaluation of CD11b staining in a region of tissue up to 100 μ m of distance from the site of injection at 3 DPI for PCL, PCL Mino or free Mino loaded and injected with hydrogel (**C; a,b**) or 15 DPI for PCL and PCL Mino loaded and injected with hydrogel (**E; a,b**). The analysis (Kruskal-Wallis test followed by Dunn's post-hoc analysis) reveals a significant reduced microglia/macrophages proliferation/activation in mice treated with minocycline, mostly when it was loaded in NPs. Data are presented as mean \pm SD. Statistical significance: (*) $p\leq 0.05$.

To further confirm that NPs loaded with minocycline were able to reduce the activation state of microglia/macrophages from the phagocytic/ameboid to a more quiescent resting state, we investigated deeply the activation of the microglia/macrophage cells by studying the expression of CD68, a specific marker of activation and phagocytosis. A clear decrease of CD68 staining could be appreciated in PCL Mino treated mice compared to mice injected with only PCL (Figure 2.4 A). Moreover, many cells containing high amount of PCL Mino NPs showed low levels of CD68 compared to either cells that did not uptake NPs or cells that uptake unloaded NPs (Figure 2.4 B). This was confirmed by the correlation analysis between CD68 staining and RhB signal for single cells, evaluated around the injury site, comparing PCL versus PCL Mino mice (Figure 2.4 C). This analysis highlighted a reduced level of CD68 staining in cells with a high RhB signal for PCL Mino treated mice (0.08 Pearson coefficient) compared to PCL treated mice (0.50 Pearson coefficient), which showed higher levels of CD68 in highly RhB positive

cells. This was confirmed also by a quantitative evaluation of the overall CD68 staining comparing PCL Mino to PCL mice around the injury site (Figure 2.4 C).

To assess the duration of deactivation and/or limited proliferation of microglia/macrophages, the effect of the treatment here proposed was analyzed up to 15 DPI. At this time of analysis, the fluorescent signal of PCL NPs is no longer detectable because of their degradation. However, a persistent reduced inflammatory state near the lesion site was found. In fact, a significant reduced staining of both CD11b and CD68 was revealed in PCL Mino compared to PCL mice, the latter used as control. This was confirmed by the quantification of CD11b staining, comparing an equivalent region of tissue from the epicenter of the lesion in both PCL (1590 ± 243 mean integrated intensity \pm SD) and PCL Mino (1081 ± 174 mean integrated intensity \pm SD) (Figure 2.4 E). In addition, a significant persistent reduced activation of microglia/macrophages was demonstrated again by evaluation of CD68 staining for PCL Mino (141 ± 54 mean integrated intensity \pm SD) compared to PCL mice (721 ± 167 mean integrated intensity \pm SD) (Figure 2.4 E, b). Furthermore, an ELISA assay was carried out on SC tissue homogenates at 15 DPI, which showed significantly reduced levels of the proinflammatory cytokine IL-6 in the epicenter of the lesion in PCL Mino (26.6 ± 5.2 mean pg/mL \pm SD) compared to PCL mice (69.6 ± 38.4 mean pg/mL \pm SD) (Figure 2.4 E, a). Conversely, at this time of analysis, IL-1 β and TNF α cytokines did not show any significant difference between

the epicenter and a more peripheral portion of tissue in the rostral direction used as a control (data not shown), suggesting no relevant involvement of these cytokines during the progression of the degeneration in the traumatized site in this specific model.

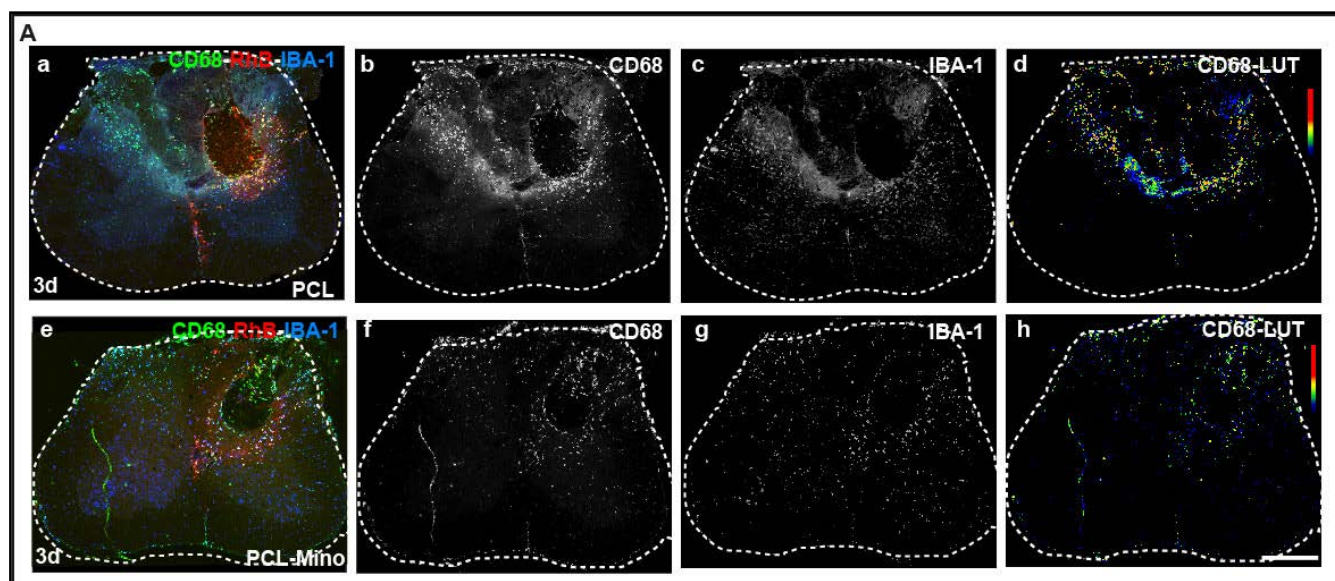


Figure 2.4 (A) PCL Mino NPs are able to reduce the pro-inflammatory milieu after trauma *in vivo*. Dashed lines outline representative low magnification of coronal sections of mice spinal cord injected with PCL NPs loaded hydrogel (a_d) or PCL Mino NPs (e_h). CD68 staining shows a lower signal in PCL Mino treated mice (e,f,h) compared to control mice (a,b,d). Activated microglia/macrophages cells, CD68 (green); PCL or PCL Mino, RhB (red); microglia, Iba-1 (blue). Scale bar=150 μ m.

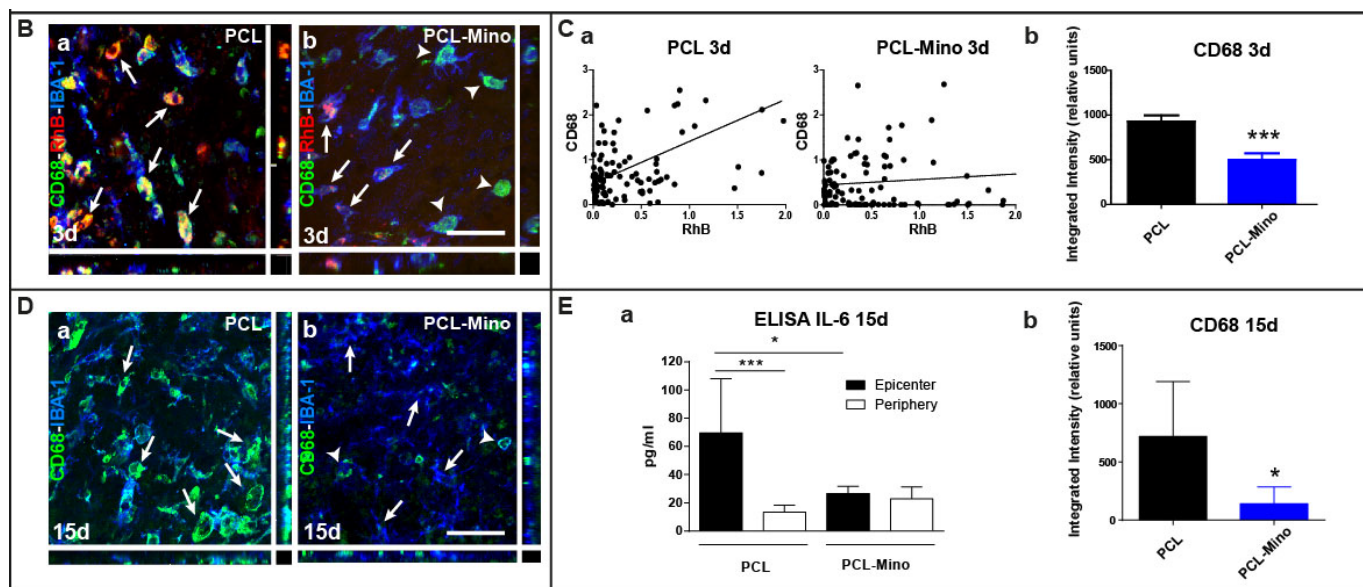


Figure 2.4 (B) High magnification of confocal image stack shows that microglia/macrophages with high level of PCL Mino NPs internalized exhibits low level of CD68 staining after 3 DPI (**b**, arrows) compared to microglia/macrophages that did not uptake NPs (**b**, arrowheads) or microglia/macrophages that uptake PCL (**a**, arrows). (**D**) This effect is evident up to 15 DPI (**a,b**) where an increased number of round shape cells with high level of CD68 is found in PCL NPs treatment (**a**, arrows) compared to PCL Mino treated mice (**b**, arrowheads). CD68 (green); PCL or PCL-Mino, RhB (red); microglia, Iba-1 (blue). Scale bar=50 μ m. (**C**) (**a**) Correlation analysis between CD68 staining and RhB signal, sampling single cells, at 3 DPI. A reduced correlation is evident for PLC Mino treated mice compared to PCL. This is represented by different linear regression slope between them suggesting an effect of minocycline in reducing the CD68 level in microglia/macrophages after PCL Mino uptake. (**C**, **E**) Quantitative analyses of CD68 staining respectively at 3 (**C**, **b**) or 15 DPI (**E**, **b**) show that a significant reduced staining of CD68 persists up to 15 DPI. (**E**, **a**) ELISA assay on spinal cord tissue homogenates shows a significant reduced level of cytokine IL-6 in the epicenter of the lesion in PCL Mino compared to PCL treated mice at 15 DPI.

Data are presented as mean \pm SD. Statistical significance: (*) $p\leq 0.05$, (***) $p\leq 0.001$.

2.2.5 Discussion and Conclusions

In this part of the work we characterized a PCL NPs delivery tool able to release, with a cell-specific targeting, a well-known anti-inflammatory drug (minocycline) in the activated microglia/macrophages. Specifically, the loading of PCL NPs with minocycline is able to reduce, in an inflammatory milieu *in vivo*, the activation and the proliferation of microglia/macrophages around the lesion site, turning them from a round shape phagocytic-like phenotype with high CD68 level to a more arborized resting phenotype with low CD68 staining.

Round shape phenotype and increased high CD68 level were well characterized in different inflammatory milieu as markers for activated microglia/ macrophages, and a reduced expression of them is considered predictive of the efficacy of anti-inflammatory treatments. In addition, the treatment here proposed limits, up to 15 days tested, the pro-inflammatory stimuli associated with microglia/macrophages activation, as demonstrated by the reduced expression of the pro-inflammatory cytokine IL-6 in traumatized site.

Furthermore, the selective and specific treatment of the microglia/macrophages here proposed shows different advantages compared to the conventional anti-inflammatory administration: (I) a drug delivery nanocarrier capable of directly targeting and modulating

specific cells characterized by a pro-inflammatory phenotype during the subacute progression of the secondary injury in SCI; (II) a targeted delivery approach, as here proposed, is able to improve and maximize the therapeutic efficacy of anti-inflammatory compounds, by reducing the drug dose used in therapy and minimizing potential side effects; (III) a selective treatment in such a specific way represents a new potential tool able to modulate at different time during the secondary injury, with temporally defined NPs administration, the activation of the microglial cells, enhancing the beneficial role of the microglia during the progression of the disease.

2.3. Evaluation of Minocycline-loaded PCL NPs efficacy in SCI mouse model

2.3.1 Aims

In order to demonstrate the increased efficacy of minocycline loaded in PCL NPs and to deeply understand the potential role of the inflammatory response related to activated microglia/macrophages and its contribution during the progression of SCI, a targeted in situ modulation of microglia/macrophages activation in SCI mouse model was carried out. In particular, different time points of treatment will be evaluated, in order to describe the detailed spatial and temporal changes of the pro-inflammatory response associated to

microglia/macrophages activation and their implication in injury progression.

2.3.2 Behavioral evaluation of SCI mice treated with PCL Mino

In order to evaluate the efficacy of the PCL Mino treatment at different time points during the inflammatory response, we performed *in vivo* experiments treating mice immediately after SCI (T0), 1 DPI (T1) or 7 DPI (T7). Specifically, mice were subjected to SCI and randomly divided in 8 groups: untreated (INJ), treated with phosphate buffered saline (saline) at T0, treated with empty NPs (PCL) at T0, treated with both free Minocycline and empty NPs (Mino free) at T0, treated with Minocycline administered intraperitoneally (Mino IP) and treated with Minocycline loaded NPs (PCL Mino at T0, T1 and T7). Six injections were performed in the injury site with a glass capillary to deliver each treatment described above (Fig 3.1 A), except Mino IP. Behavioral assessment was performed weekly starting from 7 up to 63 DPI. Locomotor performance was assessed by Basso Mouse Scale (BMS, score 0 represents complete paralysis whereas score 9 complete mobility). A physiological recovery up to about 3 BMS score at 63 DPI was detected after an initial complete paraplegia (Fig 3.1 B and C). The microinjections of saline (saline) or empty NPs treatment (PCL) in the injury site did not modify the course of the SCI progression when

compared to the untreated (INJ) group up to 63 DPI (Fig. 3.1 B). Interestingly, when we analyzed PCL Mino T0 group (28 DPI 3.7 ± 0.4 , 35 DPI 3.8 ± 0.4 , 42 DPI 4.2 ± 0.4 , 49 DPI 4.3 ± 0.4 , 63 DPI 4.3 ± 0.4 ; mean \pm SEM), we detected a significant behavioral improvement compared to untreated INJ (28 DPI 2.3 ± 0.3 , 35 DPI 2.6 ± 0.3 , 42 DPI 2.7 ± 0.3 , 49 DPI 2.9 ± 0.2 , 63 DPI 2.8 ± 0.2 ; mean \pm SEM) and Mino free groups (28 DPI 2.2 ± 0.4 , 35 DPI 2.3 ± 0.3 ; 42 DPI 2.6 ± 0.3 ; 49 DPI 2.7 ± 0.3 ; 63 DPI 2.8 ± 0.3 ; mean \pm SEM), that appears at 28 DPI and persists up to 63 DPI evaluated (Fig. 3.1 C). Contrarily, the delayed PCL Mino delivery (T1-T7) was not able to significantly improve behavioral functions when compared to untreated mice (Fig. 3.1 C). Altogether, these data suggest that PCL Mino efficacy following SCI decreases when delayed. Of note, the Mino IP treatment did not improve behavioral functions compared to the untreated INJ group (Fig. 3.1 C).

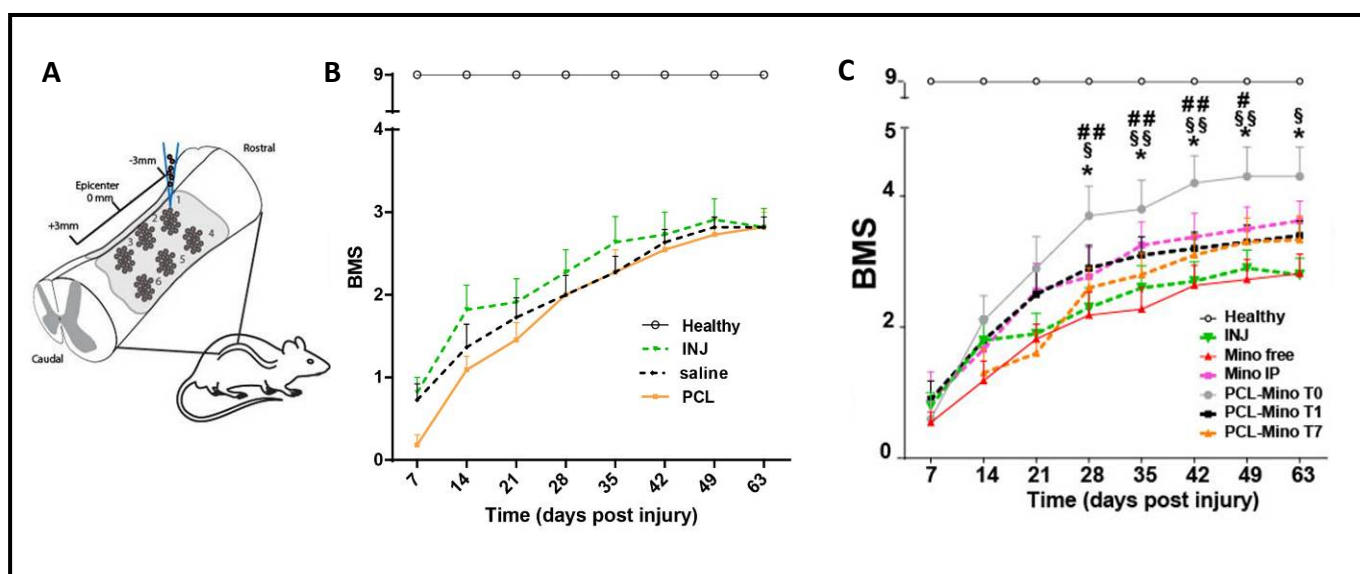


Figure 3.1 An early treatment of activated microglia/macrophages with PCL Mino NPs ameliorates the behavioral outcome in SCI mouse model. **(A)** Cartoon representing microinjections of PCL Mino NPs encompassing 6 mm around the injury site. **(B) (C)** Different SCI mice were evaluated weekly starting from 7 days post treatment by Basso Mouse Scale (score of 0 represents complete paralysis, score of 9 represents complete mobility referred to healthy mice): INJ (untreated), saline (treated with phosphate buffered saline), PCL (treated with empty NPs), Mino free (treated with both free delivered Minocycline and empty NPs directly injected into the injury site), Mino IP (treated with free delivered Minocycline intraperitoneally injected), PCL Mino T0 (treated with PCL Mino NPs injected some minutes after the mechanical damage), PCL Mino T1 (treated with PCL Mino NPs injected 1 day after the mechanical damage), PCL Mino T7 (treated with PCL Mino NPs injected 7 days after the mechanical damage). A significant improvement of locomotor performances in PCL Mino T0 treated mice is evident starting from 28 DPI up to 49/63 DPI when compared to INJ (*), Mino free (§) or PCL Mino T7 (#) groups. (*, §, #) $p \leq 0.05$; (**, §§, ##) $p \leq 0.01$. Mean \pm SEM is reported. N=12.

2.3.3 Evaluation of PCL Mino selective uptake in SCI mice

In order to confirm the selective uptake of PCL Mino NPs in SCI mice, an *ex vivo* staining with markers for astrocytes (glial fibrillary acidic protein, GFAP), neurons (neuronal nuclei, NeuN) or oligodendrocytes (myelin basic protein, MBP) was performed, while microglia were detected by CX3CR1-GFP expression. At 3 DPI, an inflammatory reaction was detected in the injury site with an increased microgliosis (Fig. 3.2 A, green) compared to healthy mice (data not shown).

Furthermore, hypertrophic astrocytes were found around the lesion (Fig. 3.2 A, c blue). In this inflammatory scenario a distribution of PCL Mino was found in and around the injury site, revealing a selective uptake of NPs in the nearest activated-phagocytic microglia (Fig. 3.2 A, d,h,i merged channels). A lack of colocalization between NPs and GFAP or NeuN or MPB was detected (Fig. 3.2 A, merged channels; Fig. 3.2 B). The selective uptake of RhB positive NPs by microglia was further confirmed by the colocalization analysis demonstrating a significant overlap between GFP and RhB signals, as demonstrated by positive Pearson's coefficient (range 0-1 from lack to complete merge) for GFP and NPs, whereas a lack of colocalization was detected for NPs and astrocytes or neurons or oligodendrocytes (Fig. 3.2 B).

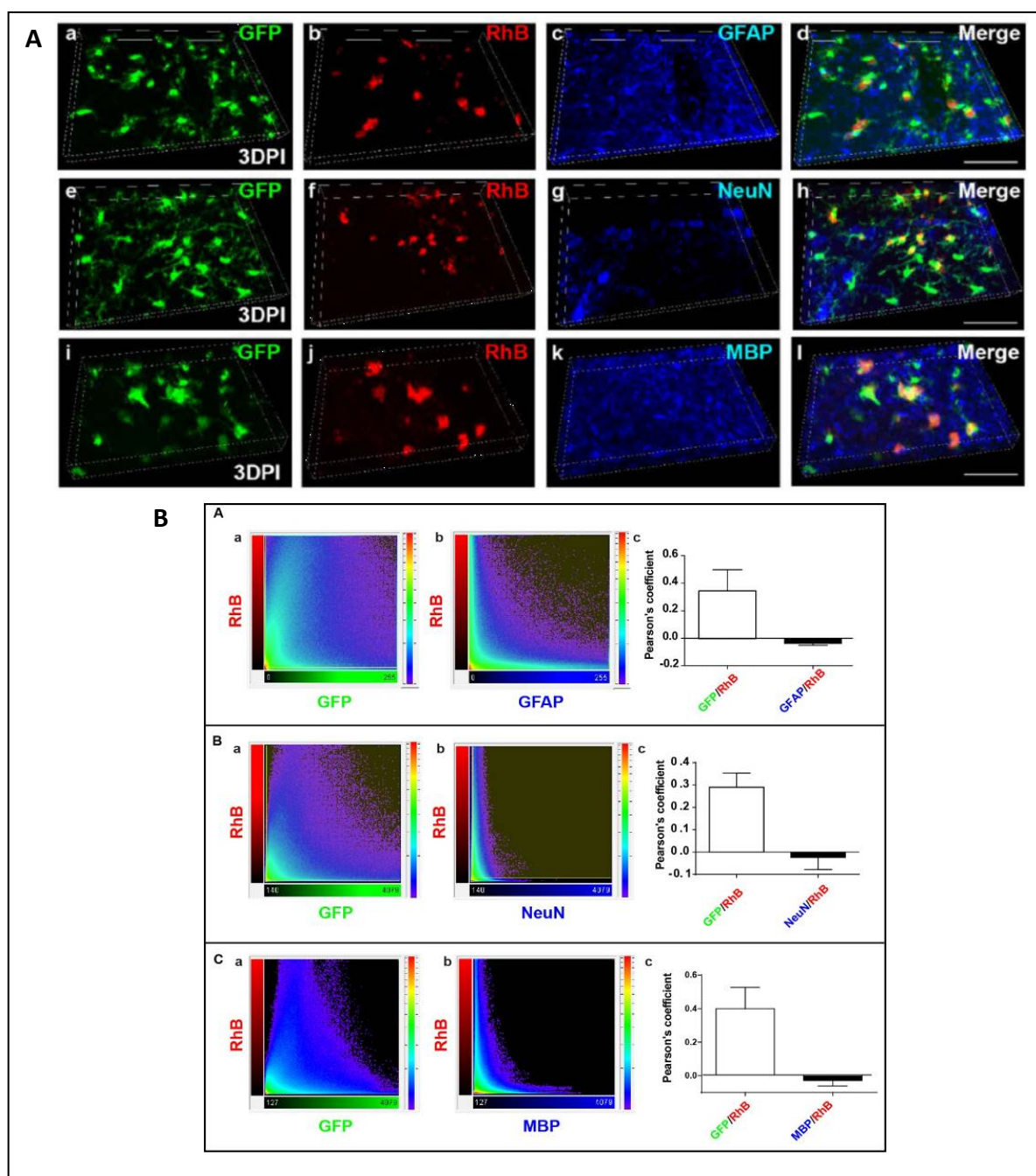


Figure 3.2 (A) PCL Mino are selectively taken up by activated microglia/macrophages *in vivo* after intraparenchymal injections in the injury site. Representative high magnifications of the injury site after injection of PCL Mino NPs T0 at 3DPI. Images show a colocalization between PCL Mino NPs (RhB, red) (**b, f, j, d, h, l**) and CX3CR1-GFP positive microglia (GFP, green) (**a, e, i, d, h, l**), but not with astrocytes (GFAP, blue) (**c, d**) or neurons (NeuN, blue) (**g, h**) or oligodendrocytes (MBP, blue) (**k, l**), revealing a selective PCL NPs uptake by activated microglia. Scale bar=50 μ m.

(B) Representative colocalization analysis by dot blot pixel **(A,B,C; a,b)** and Pearson's coefficient **(A,B,C; c)** of spinal cord coronal sections of the injury site at 3DPI after injection of PCL Mino NPs T0. Pictures show a colocalization of NPs (RhB, red) **(A,B,C; a, b, c)** only with CX3CR1-GFP positive microglia (GFP, green, **A,B,C; a, c)**, but not with astrocytes (GFAP, blue) **(A; b,c)** or neurons (NeuN, blue) **(B, b,c)** or oligodendrocytes (MBP, blue) **(C, b,c)**. Mean \pm SD is reported.

In order to further investigate the selective uptake of NPs by myeloid cells, we specifically evaluated both microglia and recruited macrophages capability to capture PCL at different time points after SCI. Six injections of PCL Mino were performed (Fig. 3.1 A) immediately after SCI (corresponding to the time point showing therapeutic effects) and both microglia and recruited macrophages were evaluated by FACS analysis at 1 and 7 DPI. To distinguish between the two cell populations, we performed a double labeling with CD11b and CD45 antibodies, identifying microglia by a CD11b^{pos}/CD45^{low} signal and recruited macrophages by a CD11b^{pos}/CD45^{high} signal, according to the literature (Shechter 2013, Alexander 2009). In response to SCI, the amount of resident microglia increased very early at 1 DPI up to 7 DPI (data not shown). At variance, recruited macrophages were barely detected in the injury site at 1 DPI and peaked at 7 DPI (data not shown). Interestingly, PCL Mino uptake at 1 DPI was high in microglia cells while very low in recruited macrophages (Fig. 3.3 a). At 7 DPI PCL Mino (Fig. 3.3 b) were absent in microglial cells, due to the degradation as previously demonstrated both *in vitro* and *in vivo*, and still very low

in recruited macrophages (Fig. 3.3 b). Quantitative evaluation expressed as NPs uptake ratio between microglia and recruited macrophages confirmed the predominant RhB signal in microglial cells at 1 DPI (Fig. 3.3 c).

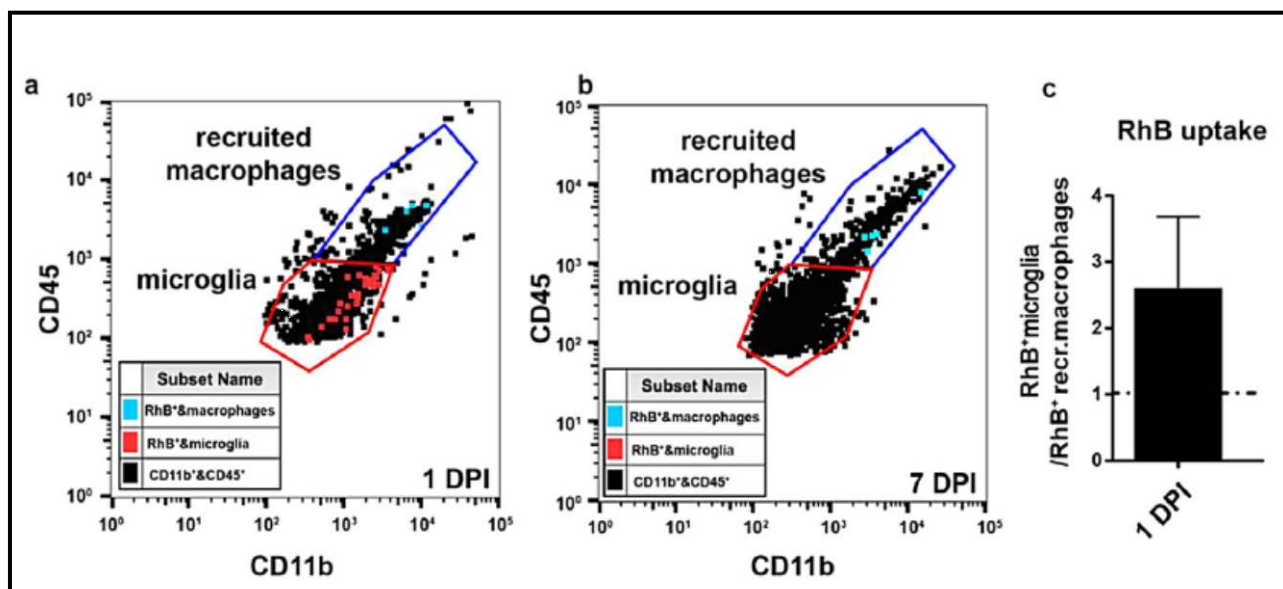


Figure 3.3 PCL Mino TO NPs are taken up early by activated resident microglial cells in SCI mouse model. Representative biparametric flow cytometric analysis of spinal cord resident microglial cells (CD11b^{pos}/CD45^{low}) and monocytes/macrophages (CD11b^{pos}/CD45^{high}) evaluated at 1 **(a)** or 7 **(b)** DPI. Black dots represent the overall population of microglia and recruited macrophages positive for CD11b and CD45 markers. Red and blue dots represent, respectively, microglia or recruited macrophages that uptake PCL Mino NPs (RhB). **(c)** Quantitative evaluation of PCL Mino NPs uptake at 1 DPI, expressed as ratio of RhB positive microglia/RhB positive recruited macrophages at 1DPI. Mean±SD is reported.

2.3.4 Evaluation of microgliosis and macrophages recruitment after PCL Mino treatment in SCI mice

We assessed by FACS analysis whether the administration of PCL Mino was able to modulate activated resident microglia and recruited macrophages in the injury site. The amount of CD11b/CD45 positive labeled cells was measured by FACS analysis in the injury epicenter. Mice treated with PCL Mino T0 and analyzed at 7 DPI (corresponding to the peak of microglia activation and recruited macrophages migration in the injury site in our model) showed a significant reduced amount of both microglia and recruited macrophages in the injury site (Fig. 3.4 b) compared to untreated INJ mice (Fig. 3.4 a), as demonstrated by dot plots (Fig. 3.4 a, b) and quantitative analyses (Fig. 3.4 c, d). These data highlight the ability of PCL Mino T0 treatment in reducing inflammation associated to microgliosis and macrophages infiltration.

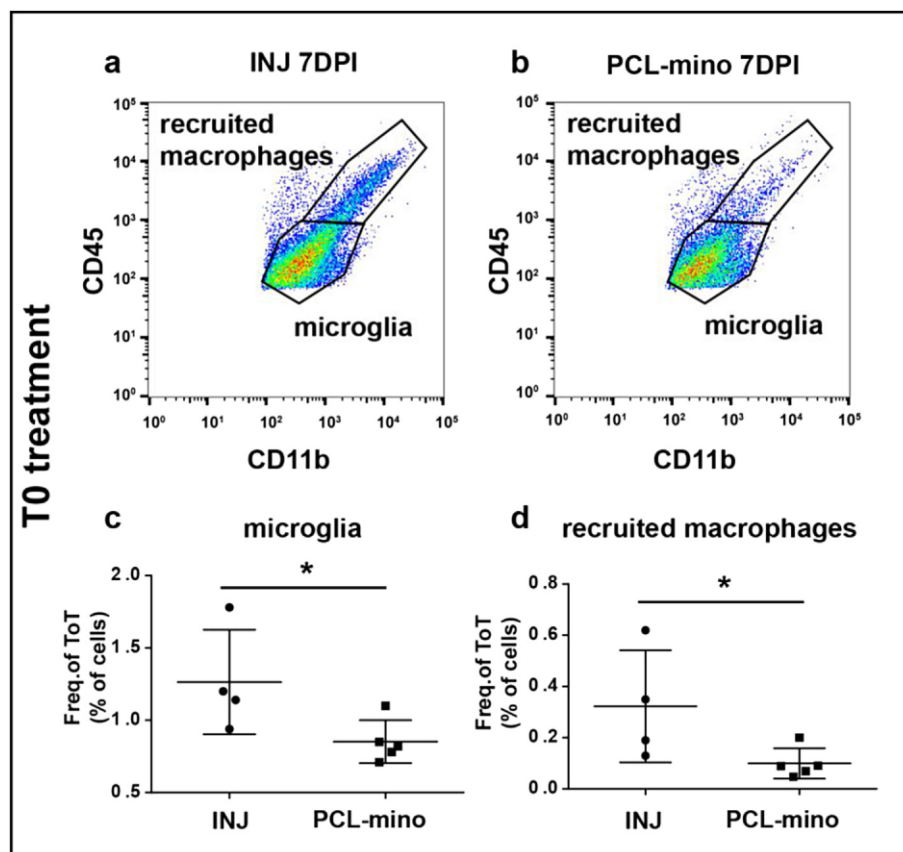


Figure 3.4 An early treatment with PCL Mino inhibits microglia activation and macrophages recruitment in the injury site of SCI mice. Representative FACS dot plot analysis of spinal cord microglial cells (CD11b^{pos}/CD45^{low}) and monocytes/ macrophages (CD11b^{pos}/CD45^{high}) present in SCI epicenter of untreated injured **(a)** or PCL Mino T0 treated injured mice **(b)** evaluated at 7 DPI. Quantitative evaluation of the percentage of CD11b^{pos}/CD45^{low} (microglia) **(c)** or CD11b^{pos}/CD45^{high} (recruited macrophages) **(d)** cells evaluated in untreated injured (INJ) or PCL Mino T0 treated injured mice (PCL Mino) at 7 DPI. Mean \pm SD is reported. (*) $p \leq 0.05$.

2.3.5 Evaluation of M1/M2 subsets after PCL Mino treatment in SCI mice

To evaluate whether the proposed treatment was able to modulate both M1 and M2 subsets in the injury site, FACS analyses were performed to detect the expression of the surface markers for M1 (CD11b^{pos}/CX3CR1^{low}/Ly6C^{high}) or M2 (CD11b^{pos}/CX3CR1^{high}/Ly6C^{low}) (Shechter 2013) phenotypes. Quantitative evaluation demonstrated an accumulation of both M1 and M2 cells in the epicenter of the lesion starting from 1 up to 7 DPI. Specifically, we detected an earlier accumulation of M1 subset in the injury site that reached a peak at 3 DPI and decreased at 7 DPI (data not shown). On the other hand, a significant amount of M2 cells in the injury site peaked at 7 DPI (data not shown). This suggests the presence of an early migration of M1 macrophages that partially decreased in favor of an M2 subset later on. Interestingly, PCL Mino T0 treatment (Fig. 3.5 A; b, c) significantly reduced the amount of M1 macrophages detected in the epicenter of the lesion at 7 DPI compared to untreated INJ mice (Fig. 3.5 A; a, c). This was also confirmed by a reduction of both IL-1 β and TNF α (two well-known M1 markers) detected in the epicenter of PCL Mino treated mice at 3 DPI by respectively mRNA and ELISA techniques (Fig. 3.5 B; a, b). Furthermore, TNF α levels in the injury site after PCL Mino T0 treatment remained comparable to the uninjured rostral portion (cervical tract) of the same mice analyzed in the epicenter (Fig. 3.5 B, b). Noteworthy, an M2 subset persisted in PCL Mino mice treated

immediately after SCI as demonstrated by the CD11b^{pos}/CX3CR1^{high}/Ly6C^{low} population detected in the epicenter of the lesion at 7 DPI (Fig. 3.5 A; a, b, d). Accordingly with a pro-regenerative milieu, mRNA evaluation in PCL Mino T0 treated mice demonstrated a significant augmented M2 transcripts of both Arg-I and YM1 at 3 DPI compared to untreated injured mice (Fig. 3.5 B; c, d). These data suggest that M2 phenotype cells were longer available compared to M1, thus fostering healing processes (Fig. 3.5 A, B). Contrarily, persistent M1 (TNF α mRNA) and M2 (Arg-I and YM1 mRNA) subsets were found in PCL Mino T7 treated mice evaluated 3 days after treatment (10 DPI) (data not shown).

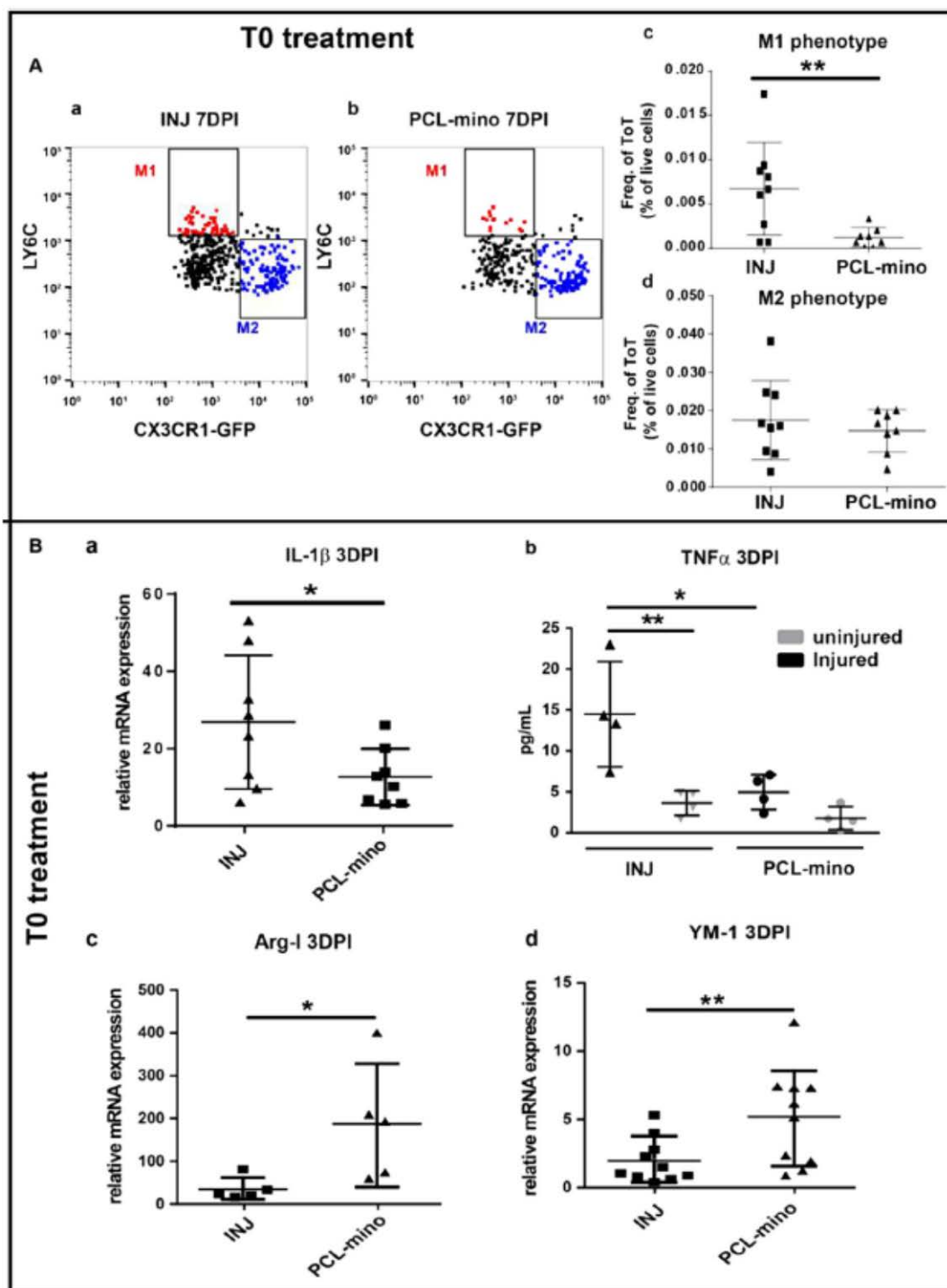


Figure 3.5 M1 recruited macrophage population is reduced after an early PCL Mino treatment in SCI mouse model. **(A)** Representative FACS dot plot analysis of M1/M2 macrophagic cells present in the injured site of untreated injured **(a)** or PCL Mino treated **(b)** SCI mice and gated respectively as CD11b^{pos}/CX3CR1-GFP^{low}/Ly6C^{high} (M1, red) or CD11b^{pos}/CX3CR1-

GFP^{high}/Ly6C^{low} (M2, blue) at 7 DPI. The percentage of M1 positive gated cells is significantly reduced in PCL Mino T0 treated mice compared to injured mice (**c**), whereas M2 subset is not significantly changed (**d**). (**B**) The quantitative analysis of mRNA or protein levels of M1 (IL1 β , **a** and TNF α , **b**) or M2 markers (Arg-1, **c** and YM1, **d**) at 3 DPI confirms that PCL Mino T0 treatment is able to preserve and promote an M2 phenotype (**c,d**) and to reduce a pro-inflammatory M1 macrophage population (**a,b**) when compared to untreated injured mice (INJ) (**a, b, c, d**). Mean \pm SD is reported, (*) $p\leq 0.05$, (**) $p\leq 0.01$.

2.3.6 Evaluation of CCL2 levels in the injury site of PCL Mino treated SCI mice

In order to evaluate potential chemoattractant stimuli that induce macrophages infiltration into the injury site, we investigated a specific chemokine CCL2 that is involved in proinflammatory M1 macrophages recruitment from the blood stream (Shechter 2013). In the *ex vivo* injured tissue, we observed a significant reduction of both mRNA and protein levels of CCL2 after PCL Mino T0 treatment (respectively 82.2 ± 65.3 relative mRNA expression, mean \pm SD; 47.0 ± 2.1 pg/mL, mean \pm SD) compared to untreated mice (respectively 138.0 ± 81.4 relative mRNA expression, mean \pm SD; 60.0 ± 8.2 pg/mL, mean \pm SD) at 3 DPI (Fig. 3.6 A; a, b). Furthermore, no significant change for CCL2 mRNA were measured after PCL Mino T7 treatment (data not shown), confirming that only a prompt anti-inflammatory treatment after SCI has a significant healing efficacy.

To confirm that a reduced migration of M1 monocyte/macrophages occurred after PCL Mino TO treatment, we performed an *in vitro* chemotaxis assay. Specifically, macrophages were recruited from the peritoneal cavity and stimulated by TNF α +IFN γ (M1 polarization). The migration toward a reservoir containing conditioned media of: i) unstimulated microglia; ii) LPS+PCL pre-stimulated microglia; or iii) LPS+PCL Mino pre-stimulated microglia was assessed by COM (COM, Centre of Mass represents the spatial averaged point of all migrated cells) (Fig. 3.6 B; f). Noteworthy, conditioned media from LPS+PCL Mino treated microglia (Fig. 3.6 B; c, d) (COM 0.94 ± 0.55 mm, mean \pm SD) was able to significantly slow down the migration of M1 macrophages compared to LPS+PCL treatment (Fig. 3.6 B; b, d) (COM 5.32 ± 2.0 mm, mean \pm SD), here considered as a control. At variance, conditioned media from untreated microglia induced a limited M1 macrophages migration (Fig. 3.6 B; a, d) (COM 0.22 ± 0.08 mm, mean \pm SD). To further confirm that CCL2 was involved in the chemotactic response, we measured the CCL2 levels in the conditioned media by ELISA, and we found that PCL Mino treatment of LPS stimulated microglia significantly reduced the paracrine CCL2 release (860.9 ± 143.9 pg/mL, mean \pm SD) when compared to the treatment with LPS+empty PCL (1268.0 ± 407.3 pg/mL, mean \pm SD) (Fig. 3.6 B e). These results demonstrate that a reduced recall of pro-inflammatory macrophages via CCL2 occurs when Minocycline-containing PCL were

added, and that untreated microglia can orchestrate and exacerbate an additional inflammatory response.

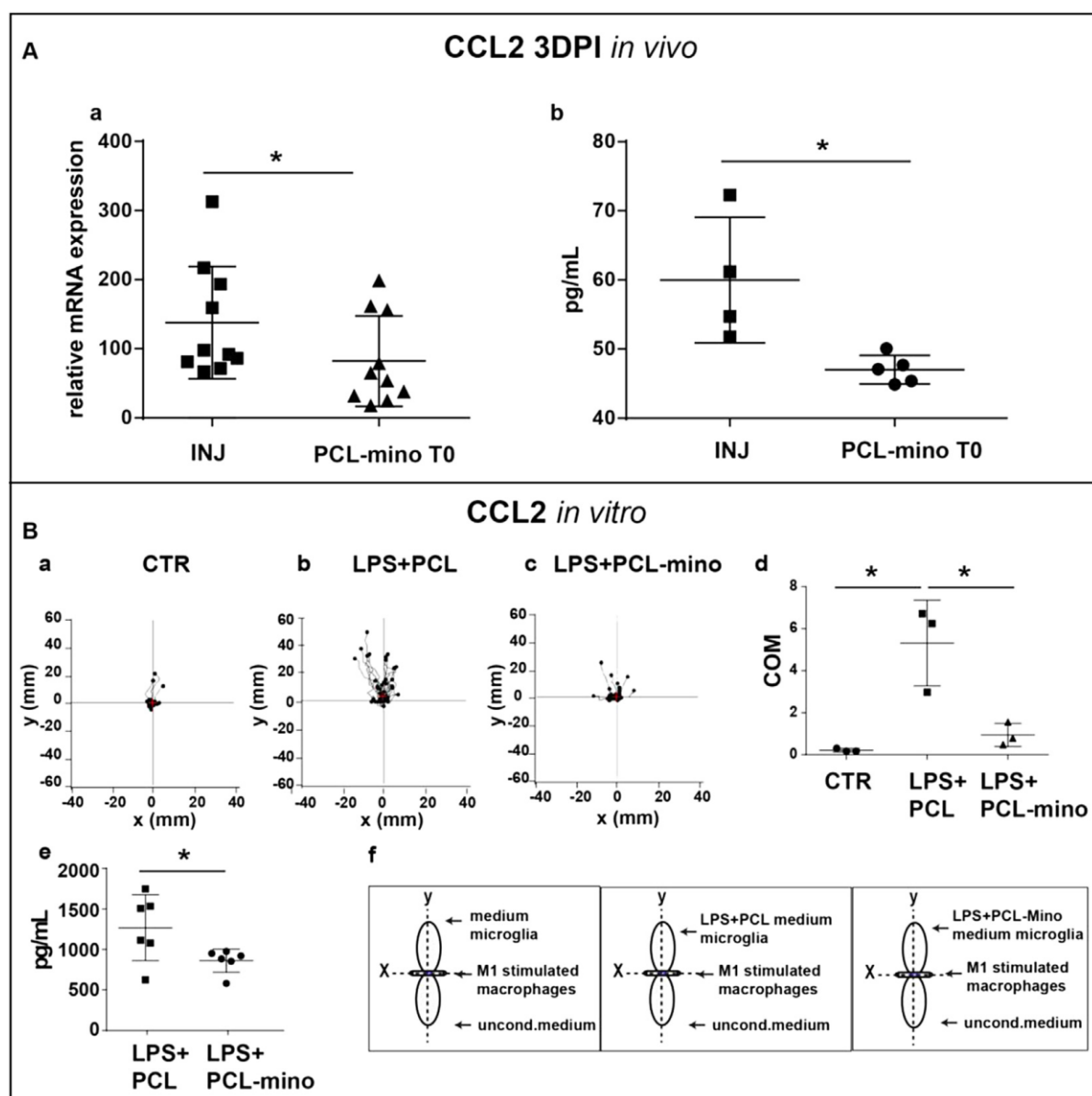


Figure 3.6 (A) CCL2 mRNA and protein levels in untreated injured or PCL Mino T0 treated injured mice evaluated at 3DPI **(a,b)**. A significant decrease of both mRNA and protein levels was detected in PCL Mino T0 group compared to untreated injured mice **(a,b)**. Mean \pm SD is reported. (*) $p < 0.05$. **(B)** An early treatment with PCL Mino inhibits CCL2 mediated M1 recruited macrophage migration. In vitro chemotactic assay: M1 stimulated

macrophages migration was tested using conditioned media of untreated (non-activated), LPS+PCL or LPS+PCL Mino treated microglia such as represented by the cartoon **(f)**. Poor migration of M1 stimulated recruited macrophages occurs toward unconditioned medium or conditioned medium from non-activated microglia (CTR, **a**, **d**). An increased migration has been observed toward conditioned medium from activated microglia treated with PCL **(b, d)**. Treatment of activated microglia with PCL Mino decrease the migration of M1 stimulated macrophages, as shown by representative migration plot **(c)** and quantitative evaluation of center of mass (COM) **(d)**. Conditioned media of both LPS ±PCL or LPS±PCL Mino treated microglia were evaluated for CCL2 protein levels showing a significant reduced amount of CCL2 in PCL Mino treated group **(e)**. Mean±SD is reported. (*) $p \leq 0.05$.

2.3.7 Evaluation of neuropathological outcome after PCL Mino treatment in SCI mice

Motor function outcome after SCI is tightly correlated with white matter (myelin) sparing (Sundberg et al.; 2010) We thus analyzed the lesion size by using whole mount myelin staining with Light Sheet Fluorescence Microscopy (LSFM). LSFM is a recently developed technology (Dodt et al.; 2007) that allows the reconstruction of the three dimensional distribution of the fluorescent signals in a whole spinal cord, without the need of physical sectioning. This method provides a general overview of the expression of particular fluorescent markers inside the spinal cord, and allows an accurate quantification of the fluorescent signals. LSFM analysis showed a well-organized and preserved white matter in the epicenter of the lesion of PCL Mino treated mice (Fig. 7; b, c). This was confirmed by a more preserved

connection and continuity in the myelin staining in the cord detected by quantitative evaluation of the fluoromyelin labeling (Fig. 3.7; d). Differently, an extended lesion size was revealed in the epicenter of the lesion in untreated injured mice, suggesting an increased spread of the damage (Fig. 3.7; a, c, d).

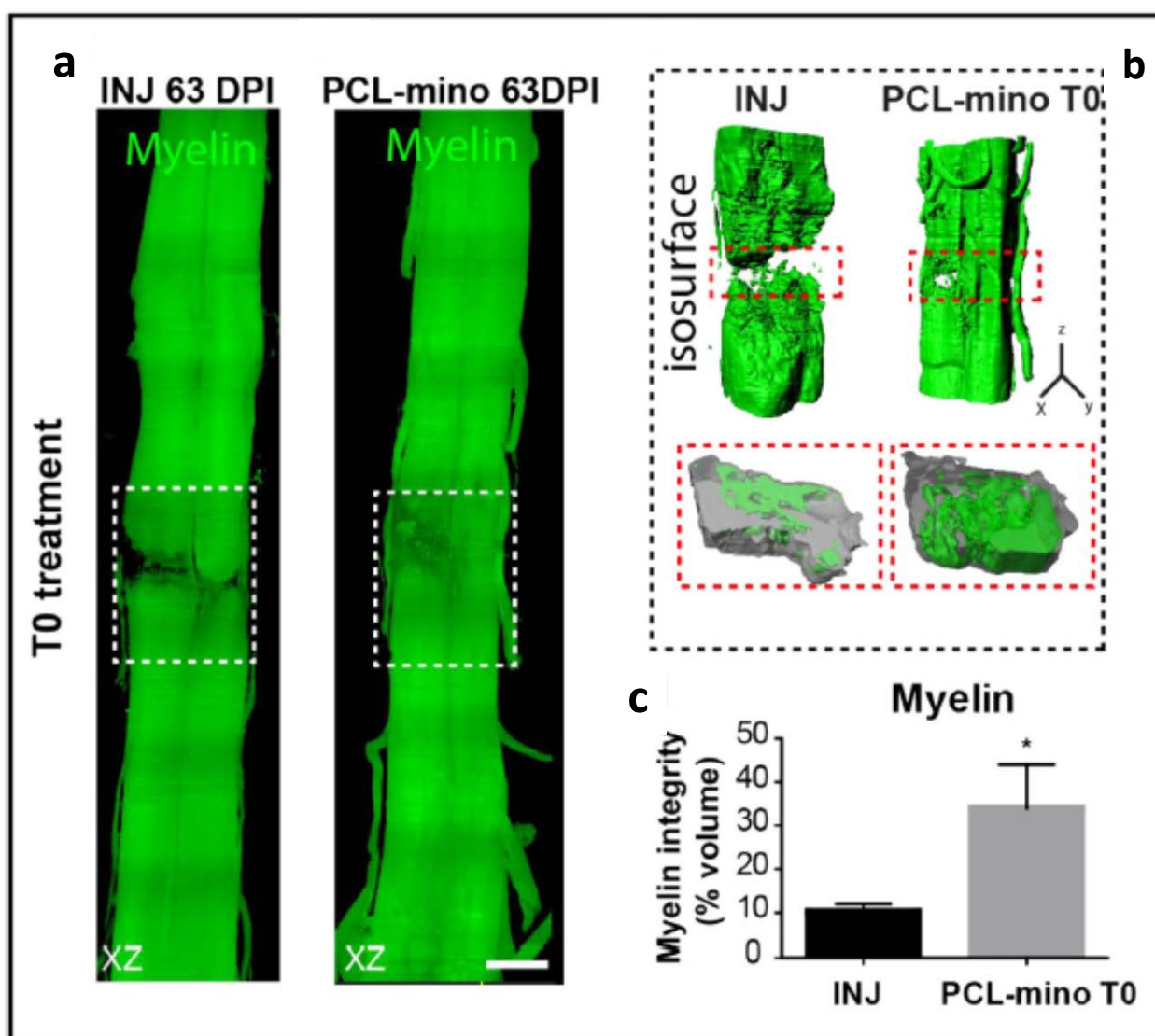


Figure 3.7 An early treatment with PCL Mino preserves white matter in the injury site of SCI mouse model. Representative longitudinal section of spinal cord of untreated injured (INJ, **a**) or PCL Mino T0 treated injured (**b**) mice at 63 DPI stained with fluoromyelin (myelin, green). Boxed regions represent the

injury site. The LSM analysis shows a more preserved myelin labeling in the injury site of PCL Mino T0 treated mice compared to untreated injured mice (**a**), as confirmed by the three-dimensional reconstruction (**c**) and by the quantification of the percentage of myelin volume preserved in the epicenter of the lesion (**d**). Scale bar=1 mm. Mean \pm SD is reported. (*) $p\leq 0.05$.

2.3.8 Discussion and Conclusions

The real contribution of the inflammatory response related to the activated state of microglia/macrophages during the progression of SCI is still debated. In this study, we used minocycline-loaded PCL NPs to pharmacologically restrain the inflammatory response associated with the microglia/macrophage activation. Many independent groups demonstrated that a range of doses of minocycline administered intraperitoneally (IP) or intravenously (IV) at different times from the injury (Festoff et al.; 2006, Teng et al.; 2004, Yune et al.; 2007) were able to ameliorate the behavioral outcome. Furthermore, it has been shown that minocycline treatment reduces the pro-inflammatory response associated with the activation of microglia/macrophages (Wells et al.; 2003). However, minocycline can act on many pathways (cytochrome C release, MAP kinase, oxidative stress) and cells within the nervous system (Teng et al.; 2004, Yune et al.; 2007). For these reasons, the direct effect on microglia remains purely speculative.

A controlled drug release through polymeric NPs as here proposed is also an innovative strategy to improve the knowledge of the role played by microglia during SCI repair overcoming the difficulties so far reported, namely: i) the unfeasibility to pharmacologically treat microglia in a selective way during SCI repair, ii) a limited drug efficacy potentially due to the widespread diffusion of the drug when administered by conventional delivery strategies (IP or IV), iii) a genetic manipulation approach that could include compensatory effects leading to misinterpretation of the involved mechanism (Wieghofer et al.; 2015). On the basis of previous evaluations, it has been demonstrated that PCL NPs were selectively taken up by microglia exploiting the peculiar phagocytic activity of these cells. Furthermore, minocycline delivered by PCL was able to effectively deactivate microglia and reduce proinflammatory cytokine release. To extend the previous findings, this smart delivery tool has been used to analyze and clarify the temporal and spatial involvement of the inflammatory microglia response during SCI. First of all, it has been demonstrated that minocycline is able to directly act on microglial cells reducing their inflammatory response. In fact, given the minocycline delivery approaches previously proposed (IP and IV) (Festoff et al.; 2006, Teng et al.; 2004, Yune et al.; 2007), the direct target associated with the therapeutic response to the treatment has remained so far unknown and only speculative. Noteworthy, it has been demonstrated also that a very early PCL Mino selective treatment is able to significantly ameliorate the deterioration of the locomotor

performance after SCI. Specifically, we found that PCL Mino treatment administered in the early phase of the injury is able to efficiently modulate resident microglial cells, reducing the pro-inflammatory milieu for several days (up to 7 days after the primary injury). By FACS analysis, it has been demonstrated that minocycline loaded nanoparticles are almost exclusively taken up by microglial cells 1 DPI. Indeed, resident microglia, corresponding to the first pro-inflammatory cells activated in the spinal cord (Thawer et al.; 2013, Cohen et al.; 2014, Raposo et al.; 2014, Beck et al.; 2012), are stimulated immediately after SCI and only when mice were treated immediately after injury with PCL Mino, is a significant improvement of behavioral outcome observed. This suggests that a dramatic effect is associated with the pro-inflammatory activation of the microglial cells in a very short time after the injury that, if not promptly deactivated, exacerbates the tissue damage. Noteworthy, macrophage recruitment is reduced after the early anti-inflammatory treatment by Minocycline, revealing that a selective modulation of the resident microglia has a key role in the recruitment of peripheral macrophages as demonstrated by a significant reduction of CD11b^{pos}/CD45^{high} cells in the epicenter of the injury. Recently, it has been demonstrated that M1 and M2 recruited macrophages reach the damaged site by distinct routes of migration (Shechter et al.; 2013). Specifically, M1 phenotype is recruited via spinal cord leptomeninges, whereas M2 commitment occurs through the passage of the choroid plexus (Shechter et al.; 2013). Furthermore, M1

and M2 show different waves of activation, with an early M1 migration in the injury site in the first days followed by M2 recruitment several days after SCI (Shechter et al.; 2013). A significant reduction of this M1 pro-inflammatory phenotype (CD11b^{pos}/ CX3CR1^{low}/Ly6C^{high} cells) in the epicenter of PCL Mino T0 treated mice has been observed by FACS analysis, whereas M2 phenotype (CD11b^{pos}/ CX3CR1^{high}/ Ly6C^{low} cells) remains in situ. In line with these results, reduced levels of both IL-1 β mRNA and TNF α protein (M1 markers) were found at 3 DPI in PCL Mino T0 treated mice, concomitantly to an increase of both Arg-I and YM1 mRNA (M2 markers), confirming a predominant pro-regenerative environment at this time-point. A reduced CCL2 level (a cytokine directly involved in the M1 recruitment) (Shechter et al.; 2013) has been observed in the injured site, suggesting that a reduced recruitment of M1 pro-inflammatory macrophages occurred in PCL Mino treated mice. Supporting this, we demonstrated by an *in vitro* chemotaxis assay that the migration of M1 pre-stimulated peripheral macrophages is significantly reduced toward PCL Mino treated compared to untreated microglia. The pro-regenerative environment established after PCL Mino T0 treatment could also explain the significant preservation of white matter in the injury site of treated mice (Doring et al.; 2015, Evans et al.; 2014, Guerrero et al.; 2012, Ma et al.; 2015). Indeed, thanks to the accurate evaluation of the entire volume of the injured site by using LSM, a milder tissue damage has been observed in PCL Mino treated mice, represented by a significant preserved myelin

staining, compared to untreated animals. In conclusion, this thesis demonstrates that early activated microglia after SCI are an important mechanistic driver of SCI associated secondary injury. Furthermore, microglia inhibitory treatment delivered immediately after injury induces a significant long lasting recovery up to 63 days post SCI, confirming the relevant pro-inflammatory imprinting given by the activated microglia in the earliest stage of the degeneration in SCI. In addition, the pharmacological delivery by polymeric NPs presents important features, namely a selective drug delivery and a maximized pharmacological activity (anti-inflammatory effect) compared to other administration routes such as direct parenchymal or IP minocycline injection. In fact, we observed that an IP Minocycline treatment with a dosage frequently proposed in the literature (Wells et al.; 2003, Stirling et al.; 2004) was not able to significantly affect behavioral recovery after SCI in mice. Whereas a significant outcome improvement with only one timely administration of the drug loaded nanovector into the injured tissue was observed. In conclusion, the modulation of the pro-inflammatory injured environment can promote reparative mechanisms and ameliorate the outcome after SCI. Indeed, the selective delivery of drugs through tailored biomaterials, as showed in this work, is a promising and innovative approach that may represent a breakthrough in manipulating microglia activation with potential beneficial impact on SCI.

I. GENERAL DISCUSSION and CONCLUSIONS

1. OVERVIEW

Improved pharmacological approaches for limiting the progressive tissue degeneration and neuronal dysfunction after an acute spinal cord injury are needed. The main focus of this thesis was the characterization of a pharmacological nanoparticle delivery tool able to treat selectively microglia/macrophages cells, key players in the inflammatory reaction that strongly characterized the SCI progression. Indeed, microglia are the first activated inflammatory cells in the site of spinal trauma, able to release pro-inflammatory stimuli and to attract macrophages from the blood stream. Recent findings have highlighted a dual role (detrimental or beneficial) related to different activated phenotypes of microglia/macrophages after the primary SCI: proinflammatory cells (M1) or anti-inflammatory/reparative cells (M2). Detrimental effects have been associated with the M1 cells such as production of many proinflammatory mediators including cytokines (IL1 β , IL-6, and TNF α), reactive oxygen species, and inducible nitric oxide synthase. M1 phenotype results already activated in an early stage after the primary injury (minutes) with a sustained prolonged effect up to months. On the other hand, M2 cells exert protective effects in terms of tissue repair and resolution of inflammation with a temporally limited effect (some days after injury). It is well characterized that M1 subset is the most prominent activated phenotype during the progression of the trauma, suggesting that a lack

in resolving the inflammation is one of the major drivers in worsening of the secondary injury of SCI. This suggests that a long-term deactivation of microglial cells after the acute phase could be a promising strategy to counteract the detrimental contributing effect of a prolonged inflammatory response associated SCI.

For this study, polymeric PCL NPs have been used and we demonstrated by *in vitro* and *in vivo* paradigms that PCL NPs are promising for the treatment of this specific cell population because they are internalized mostly by the activated form of microglia/macrophages. In particular, we proved that PCL NPs are captured exclusively by activated microglial cells in a short time (24-48 hours), thanks to their phagocytic activity of foreign bodies. PCL NPs are biocompatible and show a complete degradation by lysosomal activity after 6 days after microglial uptake. Polymeric nanoparticles, such as PCL NPs, are able to load drugs. Previously, different NPs were used and tested in SCI (Rossi et al.; 2013) and have been used to deliver compounds directly in microglial cells (e.g. Qdot nanocrystal, Minami et al.; 2012) but they present low specificity to activated microglia and great limitation with the capability to link chemically compounds, restricting this approach to a scanty number of drugs. This property makes PCL NPs an ideal pharmacological carrier for the selective treatment of activated microglia/macrophages with anti-inflammatory drugs. Among them, minocycline is one of the most promising drug with a direct active effect

on microglial cells. Their anti-inflammatory effects are mostly associated with the suppression of microglia/macrophages proliferation and activation.

PCL Mino were tested in an *in vivo* inflammatory context (hydrogel intraparenchymal injection) and demonstrated to be able to modulate the activation and the proliferation of microglia/macrophages around the injection site, up to 15 days tested, turning them from a round shape phagocytic-like phenotype with high CD68 level to a more arborized resting phenotype with low CD68 staining.

These results demonstrate that these polymeric NPs can supply optimized specific targeting of activated microglia/macrophages, *in vivo*, and can release an anti-inflammatory drug (minocycline) in situ. This represents a proof of concept of the possibility to exploit this new drug delivery tool for specifically modulating activated microglia/macrophages and the associated proinflammatory events in a SCI context.

To better understand the importance of inflammation and in particular the specific role of activated microglia/macrophages during SCI progression, we perform spinal cord intraparenchymal injection of PCL Mino in SCI mice. The in situ delivery approach as here proposed is able to improve and maximize the therapeutic efficacy of minocycline, by reducing the drug dose used in therapy and minimizing side effects. In addition, an in situ treatment is able to overcome BSCB limitations,

which occurs with other traditional route of administration. SCI inflammation is a temporally well-defined process, which could have beneficial or detrimental effect in different time of SCI progression. A specific modulation of pro-inflammatory cells (microglia/macrophages) could be useful to understand the spatial and temporal changes of inflammatory response associated to these cells population and their implication in injury progression.

In this prospective, we tested different times of PCL Mino treatment in SCI mice, starting immediately after mechanical trauma (T0), up to 7 days post injury (T7).

We demonstrate that minocycline-loaded NPs treatment is able to modulate the inflammatory milieu specifically targeting activated microglia and leads to a significant and persistent amelioration of the behavioral outcome only when acutely administered (T0) after SCI. This amelioration of behavioral outcome is observed up to 63 DPI, confirming the relevant role of activated microglial cells in the first phases of secondary injury.

We therefore show that an early modulation of the pro-inflammatory microglia by minocycline-loaded NPs is able to skew toward a reparative environment the injured tissue, preserving M2 microglia/macrophages phenotype while decreasing M1. Furthermore, we provide a mechanistic link between early microglia activation and M1 macrophages

recruitment via CCL2 that can be mitigated by acute delivery of minocycline-loaded NPs following SCI.

2. GENERAL CONCLUSIONS

SCI inflammation is one of the most important deleterious mechanisms involved in the *secondary injury*. In particular, microglia and macrophages are the most involved cells in this process but their specific role have never been elucidated because of the difficulties to selectively treat them.

In this study we used PCL NPs loaded with a well-known anti-inflammatory drug (minocycline) to understand the specific role played by these cells in the inflammatory process. Our results indicate that resident microglia, the first pro-inflammatory activated cells in the spinal cord after SCI, are strongly associated with the exacerbation of inflammatory reaction and the subsequent macrophages recruitment, playing a crucial role in the orchestration of the SCI inflammatory response. Indeed, we demonstrated that a selective microglia treatment with minocycline-loaded PCL NPs, only when administered immediately after injury before macrophages infiltration, reduces the pro-inflammatory response, maintains a pro-regenerative milieu and ameliorates the behavioral outcome up to 63 days post injury.

These results demonstrate that the selective delivery of drugs through tailored biomaterials, as showed in this work, is a promising and innovative approach that may represent a breakthrough in manipulating microglia activation with potential beneficial impact on SCI. Moreover, these data suggest that the identification of the best time to act against inflammatory response has a great importance in designing therapeutic strategies to counteract inflammation after SCI.

In addition, other diseases may also respond to such treatment. In fact many central nervous system diseases show microglia/macrophage inflammatory response, such as multiple sclerosis, Alzheimer's disease, Parkinson disease, amyotrophic lateral sclerosis, prion's disease, and epilepsy. Furthermore, this delivery strategy may be considered for other potential drugs such as molecules potentially able to induce a shift from M1 to M2 phenotype, leading to beneficial effects after the inflammatory response. Moreover, the approach here proposed, which is able to treat selectively inflammatory cells using biodegradable nanoparticles with a well-known drug (minocycline) used in different human pathologies (antibiotic activity), opens a new translational avenue to the inflammatory and pain treatment in SCI patients.

3. FUTURE PERSPECTIVES

Many preclinical studies have been proposed to find potential treatments for SCI. Unfortunately, many of them showed no relevant efficacy when translated to clinical trials (Rabchevsky et al.; 2011, Hawryluk et al.; 2008). A possible reason could be that most strategies proposed have used treatments directed toward a single pathophysiological mechanism and only few attempts to find effective combination therapies (neuroprotective and neuroregenerative) were carried out to improve the outcome (Kwon et al.; 2011, Tetzlaff et al.; 2011). Other reasons could be associated with the confined pharmacological treatment of a conventional drug administration, mainly due to the low concentration achieved in CNS (for BSCB restrictions) and/or potential unacceptable side effects of prolonged treatments. Consequently, new efforts are directed to develop innovative strategies to combine different treatments and drug delivery tools, to overcome BSCB or to perform effective drug delivery directly in SC parenchyma. A combined approach by using smart biomaterials (e.g., multipolymeric approach using NPs) could represent today's new frontiers of a tailored delivery solution to control and progressively release different drugs in either CSF or SC parenchyma. Several recent studies have investigated these aspects trying to combine the advantages of biomaterials delivery systems for multitherapeutic treatments: methylprednisolone (Kim et al.; 2009), antibodies

(Stanwick et al.; 2012) and growth factors (Piotrowicz et al.; 2006) were incorporated in NPs and then loaded into hydrogels to provide a sustained release into the final target tissue, aiming at increasing medical recovery chances.

In this perspective, an early modulation of pro-inflammatory microglia as obtained in this work could be combined with other neuroregenerative treatments, in order to define a multi-therapeutic approach able to counteract the different mechanisms that characterize *secondary injury*.

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K. LIST of
PUBLICATIONS

PUBLICATIONS ARISEN FROM THIS THESIS:

1. "Early modulation of pro-inflammatory microglia by minocycline loaded nanoparticles confers long lasting protection after spinal cord injury" Papa S et al.; Biomaterials 75 (2016); 13-24
2. "Polymeric nanoparticle system to target activated microglia/macrophages in spinal cord injury" Papa S, Ferrari R et al.; J Control Release 2014; 174 : 15-26
3. "Selective nanovector mediated treatment of activated proinflammatory microglia/ macrophages in Spinal Cord Injury" Papa S, Rossi F et al.; ACS Nano 2013, 7 (11), pp 9881–9895
4. "Modulators of microglia: a patent review"
Papa S*, Caron I*, Rossi F, Veglianesi P; Expert Opin Ther Pat., 2016